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(54) Title: HELPER-FREE, TOTALLY DEFECTIVE ADENOVIRUS FOR GENE THERAPY

(57) Abstract

A method for producing *in vivo* packaged recombinant adenovirus vectors is provided. The recombinant Ad vectors do not contain any Adenovirus genes and are therefore useful for gene therapy. The recombinant Adenovirus vectors are packaged *in vivo* using a helper virus which is itself very inefficiently packaged, providing a recombinant viral preparation with very little or no contamination with helper virus. In particular, the method makes use of a helper virus in which the packaging site can be easily excised *in vivo* by recombination mediated by a recombinase. The helper virus is also useful for the *in vivo* construction of new recombinant adenovirus vectors containing substitutions in the E1 or other adenoviral region.

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HELPER-FREE, TOTALLY DEFECTIVE ADENOVIRUS FOR GENE THERAPY

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INTRODUCTION

Background

Adenoviruses (Ads) belong to the family *Adenoviridae* and the human Ads belong to the genera *Mastadenovirus*. Human Ad infections are found worldwide. Ads were initially characterized in 1953 by Rowe *et al.* when trying to cultivate epithelial cells from the adenoids. The 47 different serotypes are grouped (A-F) according to their ability to cause tumours in newborn hamsters. Respiratory epithelial cells are the primary target for Ads *in vivo*. 5% of the acute respiratory diseases in children under the age of 5 are due to Ads. Other sites of infection include the eye, the gastro-intestinal tract and the urinary tract. Many Ad infections are subclinical and only result in antibody formation.

Three loosely defined sets of protein exist in the mature Ad: proteins that form the outer coat of the capsid, scaffolding proteins that hold the capsid together and DNA-binding proteins. The diameter of the icosahedral-shaped capsid varies from 65 to 80 nm depending on the serotype. The capsid is composed of a total of 720 hexon and 60 penton subunit proteins, 360 monomers of polypeptide VI, 240 monomers of polypeptide IX, and 60 trimeric fibre proteins.

Bound to the penton subunits and protruding from the capsid is the fibre protein which mediates the initial attachment of the virus to a target cell. Polypeptides IX, IIIa, and VI form the scaffolding which holds the capsid together. Polypeptide IX stabilizes the packing of adjacent hexons in the capsid, polypeptide IIIa spans the capsid to link hexons of adjacent faces, and polypeptide

VI connects the structural proteins to the core. The core consists of DNA associated with polypeptides V, VII, μ and the terminal protein.

Ads contain double stranded DNA as their genetic material. The base composition of the 47 characterized serotypes (Ad1-Ad47) varies in the percent G + C content and in the length of the genome (approximately 36 kb) and of the inverted terminal repeats (100-140 bp). The genome is covalently linked at each 5′ end to individual 55 kd terminal proteins, which associate with each other to circularize the DNA upon lysis of the virion.

The Ad genome is functionally divided into 2 major non-contiguous overlapping regions, early and late, based on the time of transcription after infection. The early regions are defined as those that are transcribed before the onset of viral DNA synthesis. The switch from early to late gene expression takes place about 7 hours after infection. The terms early and late are not to be taken too literally as some early regions are still transcribed after DNA synthesis has begun.

There are 6 distinct early regions; E1a, E1b, E2a, E2b, E3, and E4, each (except for the E2a-b region) with individual promoters, and one late region, which is under the control of the major late promoter, with 5 well characterized coding units (L1-L5). There are also other minor intermediate and/or late transcriptional regions that are less well characterized, including the region encoding the viral-associated (*VA*) RNAs. Each early and late region appears to contain a cassette of genes coding for polypeptides with related functions. Each region is transcribed initially as a single RNA which is then spliced into the mature mRNAs. More than 30 different mature RNA transcripts have been identified in Ad2, one of the most studied serotypes.

Once the viral DNA is inside the nucleus, transcription is initiated from the viral E1a promoter. This is the only viral region that must be transcribed without the aid of viral-encoded *trans*-activators. There are other regions that are also transcribed immediately after cell infection but to a lesser extent, suggesting that the E1 region is not the only region capable of being transcribed without viral-encoded transcription factors. The E1a region codes for more than six polypeptides. One of the polypeptides from this region, a 51 kd protein,

transactivates transcription of the other early regions and amplifies viral gene expression. The E1b region codes for three polypeptides. The large E1b protein (55 kd), in association with the E4 34 kd protein, forms a nuclear complex and quickly halts cellular protein synthesis during lytic infections. This 55 kd polypeptide also interacts with p53 and directly inhibits its function. A 19 kd *trans*-activating protein encoded by the E1B region is essential to transform primary cultures. The oncogenicity of Ads in new-born rodents requires the E1 region. Similarly, when the E1 region is transfected into primary cell cultures, cell transformation occurs. Only the E1a region gene product is needed to immortalize cell cultures.

The E2a and E2b regions code for proteins directly involved in replication, i.e., the viral DNA polymerase, the pre-terminal protein and DNA binding proteins. In the E3 region, the 9 predicted proteins are not required for Ad replication in cultured cells. Of the 6 identified proteins, 4 partially characterized ones are involved in counteracting the immune system; a 19 kd glycoprotein, gp19k, prevents cytolysis by cytotoxic T lymphocytes (CTL); and a 14.7 kd and a 10.4 kd/14.5 kd complex prevent, by different methods, E1a induced tumour necrosis factor cytolysis. The E4 region appears to contain a cassette of genes whose products act to shutdown endogenous host gene expression and upregulate transcription from the E2 and late regions. Once viral DNA synthesis begins, the late genes, coding mainly for proteins involved in the structure and assembly of the virus particle, are expressed.

Recombinant human adenoviruses have attracted much attention of late because of their potential for gene therapy and gene transfer and for protein expression in mammalian cells. First-generation recombinant adenovirus vectors most often contain deletions in the E1a and/or E1b regions. The usefulness of such vectors for gene transfer has been demonstrated in mice, cotton rats and nonhuman primates (Engelhardt *et al.* Hum. Gene Ther. 4:759-769 1993; Rosenfeld *et al.* Cell 68:143-155 1992; Yang *et al.* Nat. Genet. 7:362-369 1994). A fundamental problem encountered in using these vectors for gene therapy, however, is that deletion of the E1 sequences alone is not sufficient to completely ablate expression of other early and late viral genes or to prevent replication of the

viral DNA. Studies have indicated that these vectors express viral antigens which elicit destructive immune responses in the target cells (Yang *et al.* Proc. Natl Acad. Sci.91:4407-4411 1994; Yang *et al.* Nat. Genet. 7:362-369; Yang *et al.* J. Virol. 69:2004-2015 1995). This immune response leads to loss of transgene expression and development of inflammation. In addition, there is indication that memory-type immune responses may substantially diminish the efficiency of gene transfer following a second and subsequent administrations of the recombinant vector (Kozarsky *et al.* J. Biol. Chem. 269:1-8 1994; Smith *et al.* Nat. Genet. 5:397-402 1993). Newer recombinant adenovirus vectors contain additional disabling mutations in other regions of the adenovirus genome, for example in E2a (Englehardt *et al.* Hum. Gene Ther. 5:1217-1229 1994; Englehardt *et al.* Proc. Natl Acad. Sci. 91:6196-6200) or E3 (Bett *et al.* Proc. Natl Acad. Sci. 91:8802-8806 1994). These vectors, although they express fewer viral proteins, do not completely eliminate adenoviral protein expression and so are subject to similar immune response problems as found with the earlier vectors.

In addition to the immune response problems associated with the use of the current adenovirus-based gene therapy vectors, only relatively small amounts of foreign DNA (that is, non-adenovirus DNA) can be accommodated in these vectors due to the size constraints of adenoviral packaging. Studies have shown that adenovirus virions can package up to approximately 105% of the wild type adenovirus genome length (the wild type adenovirus genome is between 35-36 kilobases). Recombinant vectors having deletions in the E1 region typically permit the insertion of less than 5 kb of foreign DNA. Recombinant vectors having additional deletions in E3 can accommodate inserts of up to about 8 kb.

Another serious problem inherent in the use of current recombinant adenovirus-based vectors is their ability to recombine with adenoviruses from natural sources to produce infections of wild type viruses.

It would be advantageous to develop a recombinant adenovirus vector that is incapable of producing any adenovirus proteins, that can accommodate large inserts of foreign DNA and that recombines only at low frequency or not at all with other adenoviruses. The present inventor has surprisingly found that recombinant adenovirus (rAd) vectors containing as little as 600 base pairs of

adenovirus sequence can be replicated and packaged *in vivo* to produce infectious virions. Adenoviral factors necessary for the replication and packaging of the minimal rAd vectors are supplied *in trans* from a recombinant adenovirus helper vector of the present invention which is designed such that the packaging site is easily excisable *in vivo* by the use of the Cre/*lox* recombination system.

Cre/*lox* is a site-specific recombination system, originally discovered in bacteriophage P1, which consists of a recombinase protein (Cre) and the DNA recognition site of the recombinase (Hoess and Abremski in "Nucleic Acids and Molecular Biology", Eckstein and Lilley, eds., Vol. 4, p. 99 Springer-Verlag 1990). Cre (causes recombination) is a member of the Int family of recombinases (Argos *et al.* EMBO J. 5:433 1986) and has been shown to perform efficient recombination of *lox* sites (locus of X-ing over) not only in bacteria but also in eukaryotic cells (Sauer Mol. Cell. Biol. 7:2087 1987; Sauer and Henderson Proc. Natl Acad. Sci. 85:5166 1988). The Cre recombinase can efficiently excise DNA bracketed by *lox* sites from the chromosome. Two components are required for recombination: the Cre recombinase and an appropriate *lox*-containing substrate DNA. Several different *lox* sites have been identified to date, for example *lox* P, *lox* 511, *lox* 514 and *lox* Psym (Hoess *et al.* Nucl. Acids Res. 14:2287-2301 1986). The sequences of the various *lox* sites are similar in that they all contain the identical 13-base pair inverted repeats flanking an 8-base pair asymmetric core region in which the recombination occurs. It is the asymmetric core region that is responsible for the directionality of the site and for the variation among the different *lox* sites. Only *lox* sites having the same sequence are recombined by Cre. Recombination between two directly oriented *lox* sites results in excision of the intervening DNA as a circular molecule having a single *lox* site and leaves a single *lox* site at the point of excision. The intramolecular excision is in equilibrium with the reverse reaction, that is, with intermolecular insertion of a DNA molecule containing a *lox* site into the identical *lox* site remaining in the chromosome. The excision reaction is favored 20 to 1 over the insertion reaction. Recombination between two inversely oriented *lox* sites results in inversion rather than excision of the intervening DNA. Cre/*lox* has been used to remove unwanted DNA sequences from the genome (for example, selectable marker genes when no

longer needed for selection), for designing recombination dependent switches to control gene expression (Sauer and Henderson Nucl. Acids Res. 17:147 1989) and to direct site-specific integration of *lox* vectors into a *lox* site previously placed into the chromosome (Sauer and Henderson New Biol. 2:441 1990).

Relevant Literature

Early experiments showed that it was possible to create defective adenoviruses which carried substitutions of all or part of the SV40 genome in tandem. The deletions included 16% to 29%, 29% to 75% and 75% to 96%, indicating that virtually all of the Ad virus could be substituted. (For a summary of these experiments see, The Adenoviruses, Harold S. Ginsberg, ed. Plenum Press, NY, 1984.)

Bett *et al.* have described an adenovirus vector containing deletions in both the E1 and E3 regions (Proc. Natl Acad. Sci. 91: 8802-8806 (1994)). Mitani *et al.* (Proc. Natl Acad. Sci. 92: 3854-3858 (1995)) have described a recombinant adenoviral vector which is deficient in E1 and contains a 7.23 kb deletion in an essential part of the viral genome carrying L1, L2, VA and TP. A marker gene was inserted in place of the deleted adenoviral DNA and the vector was replicated and packaged after tranfection of 293 cells using a wild type Ad2 virus as a helper. The helper virus was also replicated and packaged. The packaged viruses (wild type helper virus and recombinant virus) were partially separated by repeated CsCl gradient centrifugation.

Anton and Graham (J. Virol. 69: 4600-4606 (1995)) have used Cre-mediated recombination of flanking *lox* P sites to turn on expression of a luciferase gene cloned into an adenoviral vector. The recombination of the *lox* sites resulted in the removal of a fragment of DNA between the luciferase coding sequence and the promoter. The Cre recombinase was supplied from a second adenoviral vector carrying the Cre gene under control of hCMV promoter.

U. S. Patent Number 4,959,317 describes a method for producing site-specific recombination of DNA in eukaryotic cells using Cre-mediated recombination of *lox* sites. Cre-expressing eukaryotic cells are also disclosed. WO 91/09957 describes a method for producing site-specific recombination in

plant cells using Cre-mediated recombination of *lox* sites. EP 0 300 422 describes a method for preparing recombinant animal viral vectors using Cre-mediated recombination between a *lox* P site on the virus and a *lox* P site on a plasmid.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide therapeutic recombinant adenovirus-based (therapeutic rAd) vectors for gene therapy or for expression of foreign genes in mammalian cells. The therapeutic rAd vectors of the present invention contain a minimal amount of adenovirus DNA and are incapable of expressing any adenovirus antigens, i.e. "gutless". The therapeutic rAd vectors of the present invention provide the significant advantage of accommodating large inserts of foreign DNA while completely eliminating the problem of expressing adenoviral genes that result in an immunological response to viral proteins when a therapeutic rAd vector is used in gene therapy. In particular, the therapeutic rAd vector of the present invention comprises the adenovirus inverted terminal repeats, an adenovirus packaging site, one or more *lox* sites and up to 36-38 kb of foreign DNA. By "foreign" DNA is meant any genes or other DNA sequences that do not occur naturally in adenovirus.

The ability of these therapeutic rAd vectors to accommodate such large inserts of foreign DNA (up to 38 kb) permits construction of gene therapy vectors that contain and express extremely large individual genes or polynucleotide sequences as well as multiple genes or polynucleotide sequences. The foreign DNA that can be expressed can be any polynucleotide sequences that do not occur naturally in adenovirus, including the Duchenne Muscular Dystrophy (DMD) gene, all genes involved in dopamine synthesis (e.g. tyrosine hydroxylase, GPD cyclohydroxylase), Factor VIII, Factor IX, superoxide dismutase, GM-CSF (granulocyte-macrophage colony-stimulating factor), genes involved in chronic granulomatous disease (CGD), and multiple genes, including GM-CSF in combination with other cytokines (e.g. interferons (IFN-α, IFN-β, IFN-γ), interleukins, M-CSF (macrophage colony-stimulating factor), tumor necrosis factors, growth factors (TGF-β (transforming growth factor-β) and PDGF (platelet-derived growth factor)), and including GM-CSF with MHC (major

histocompatibility complex) genes. The ability to deliver expression products from extremely large sequences or multiple sequences provides a simple and efficient delivery system.

The present invention includes the discovery that the minimum size range for the rAd vectors is from 32 kb to 38 kb, as smaller rAd vectors (<32 kb) are unstable and not efficiently packaged. This discovery of the lower size limit for packaging efficiency permits increased stability, which is important for vectors intended for gene therapy and increased production efficiency, which can reduce manufacturing costs significantly. Thus, provided herein are methods of producing a rAd vector for gene therapy, comprising constructing a therapeutic rAd vector wherein said vector ranges in total size from 32 kb to 38 kb. The vectors of the invention include both plasmids as well as packaged recombinant viral and foreign DNA.

Because the therapeutic rAd vectors of the present invention do not express any adenovirus proteins, those adenovirus proteins that are required for the replication and packaging of the therapeutic rAd vectors of the present invention are supplied *in trans* by a helper recombinant adenovirus vector (helper rAd).

It is another object of the present invention to provide a helper recombinant adenovirus vector which is useful for the preparation of *in vivo* packaged therapeutic rAd vectors. The helper rAd vector of the present invention comprises adenovirus genes which encode proteins necessary for the replication and packaging of the therapeutic rAd vectors into therapeutic rAd virus particles. The helper rAd vector of the present invention additionally comprises an adenovirus packaging site flanked by at least one set of two identical *lox* sites in direct orientation. When the helper rAd of the present invention is grown in a host cell that produces Cre recombinase, the packaging site is excised by Cre-mediated recombination between the flanking *lox* sites. Since the presence of an adenovirus packaging site is absolutely required for packaging of the DNA into adenovirus virions, removal of the packaging site by excision prevents the helper rAd vector from being packaged. One of ordinary skill in the art will understand that the host cells useful in the invention can be any cell lines susceptible to adenovirus infection and capable of expressing a recombinase capable of mediating

recombination between recombination sites. Any recombinase-expressing cell line and its corresponding recombination sites can be used, including, but not limited to, the FLP recombinase and its recombination site. See O'Gorman et al., Science 251:1351 (1991). The term recombinase includes any enzymes that mediate recombination between its corresponding recombination sites, which are nucleic acid sequences that are specifically recognized by the recombinase. One of ordinary skill in the art will readily appreciate that any examples describing cre-recombinase and lox sites can be substituted with any other recombinase and its corresponding recombination sites.

Generally, the helper rAd vector of the present invention comprises all of the adenovirus genes necessary to provide the replication and packaging functions but may contain less than all of the necessary genes if the proteins encoded by some of these adenovirus genes are supplied in other ways, for example, by the host cell. In particular, the helper rAd vector need not contain the adenovirus E1a and E1b regions if used in combination with a host cell that can supply E1a and E1b gene products. If such a cell line is used, the E1a gene promoter should be transcribed from a heterologous promoter. It is essential that the Ad packaging site in the E1a promoter enhancer is not present in the host cell line.

It is a further object of the present invention to provide a system and a method for preparing a substantially pure preparation of *in vivo* packaged therapeutic rAd vector particles. The method of the present invention comprises transfecting an appropriate host cell with therapeutic rAd vector DNA and helper rAd vector DNA. The transfected cells are cultured for a sufficient time to allow maximum production of the therapeutic rAd virus. The virus particles are harvested and used to infect fresh host cells either with or without the addition of a small amount of packaged helper rAd virus particles. The virus particles produced following infection are isolated and the infection process may be repeated. In the final step, Cre-expressing host cells are infected with the viral particles produced in the earlier infections. Infection of the Cre-expressing cells provides for selection against the helper rAd so that a substantially pure preparation of packaged therapeutic rAd vector particles is produced.

Cre-expressing mammalian host cell cultures are also provided.

Also provided are methods and systems for rapidly and efficiently generating new recombinant adenovirus vectors with substitutions in a adenoviral region. These methods and systems comprise a helper rAd vector, a replicating vector containing ITRs, and Ad packaging site, substitute DNA and a recombination site identical to at least one of the recombination sites in the helper rAd vector, and a recombinase-expressing host cell line. One of skill in the art will appreciate that the vectors can be either recombinant adenoviruses or plasmids. These methods and systems provide a simple and efficient alternative to existing overlap recombination techniques. A working stock of virus can be produced for initial experiments within 10 days. Moreover, substituted rAds can be generated in substantially pure form without need for plaque purification. This solves the problem of purifying the recombinant adenovirus vectors with substitutions away from the rAd helper vectors. Other advantages of these methods are that (a) viral sequences in plasmids are more stable and easier to prepare than viral DNA, which is under continuous selection during growth and (b) recombinase-mediated recombination enables use of a small replicating vector which is extremely easy to manipulate. One of ordinary skill in the art will appreciate that both replicating and nonreplicating vectors can be used in these methods and systems.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. The genetic map of a wild type Adenovirus.

FIG. 2. Nucleotide sequence of several different *lox* sites.

FIG. 3. Schematic representation of the helper rAd helper vector Ψ5. The bottom figure represents the complete Ψ5 genome. The upper figure shows the details of the structure of the insert carrying the packaging site flanked by the lox P sites which is substituted into the Ad5 at position 0-3328.

ITR = inverted terminal repeat; P_{CMV} = CMV promoter; P_{E1a} = E1a promoter; arrow following ITR indicate the orientation. The vertical lines to the right of the P_{CMV} indicate restriction sites in the polylinker.

FIG. 4. Construction of a 'gutless' adenoviral vector. In the first step shuttle plasmid cleaved at the ITR is transfected into CRE8 cells with Ψ5 DNA.

The proteins from Ψ5 convert the shuttle plasmid DNA to a molecule which is replicated by adenovirus DNA polymerase. The gutless virus is then encapsidated into adenovirus capsids. Several rounds of growth are necessary to amplify the gutless virus. At each round, more Ψ5 virus is added to insure that all cells contain the helper virus.

FIG. 5. is a gel showing a restriction analysis of packaged gutless and Ψ5 DNA from infected CRE8 cells. The DNA was digested with Bgl II. There are no Bgl II sites in the loxAβ gutless virus. The lanes contain: M, 1 kb ladder + 16.5 and 33.5 kb fragments; Ψ5 DNA; lanes 1-6, isolates of loxAβ + Ψ5. The arrow marks the position of loxAβ DNA.

FIG. 6. is a gel showing a restriction analysis of loxAβ + Ψ5 DNA with ClaI. Ψ5 DNA contains one site at base 1473. The predicted sizes of the loxAβ fragments are 0.5, 1.2, 6, 7.5, and 11.4 kb. The lanes contain: M, 1 kb ladder; P, ploxAβ cut with ClaI; isolates 2, 5 and 6 from part a cut with ClaI. (Many of the bands from ploxAβ do not match up with their cognate bands in loxAβ as the ClaI sites in the plasmid are methylated.)

FIG. 7. Restriction analysis of Ψ5 mutants with BsaBI. The position of the left end fragment from Ψ5 is marked. The DNA was from 16 plaque isolates purified on 293 cells.

FIG. 8A-8C. Methods for selecting gutless viruses. These methods allow for enrichment of gutless virus starting with a mixture containing about 0.5 % gutless, and going to roughly 50 %. In each arrangement, the gutless virus will be enriched based on expression of a gene in the virus. This selectable marker is flanked with lox511 sites and will be deleted from the gutless virus by growth in CRE8 cells for the final step of enrichment. The final enrichment takes the gutless virus concentration to 95% or more. ITR is the inverted terminal repeat of an adenovirus, Ψ is the packaging site of an adenovirus. loxP and lox511 are loci of Cre recombinase directed recombination. loxP will recombine with loxP but not lox511 and visa versa.

FIG. 8A. Sorting for an expressed gene on the gutless virus. Here the selection is accomplished by mechanical means such as a cell sorter or by panning with an antibody. Ψ5 is used as a helper virus. Ψ5 DNA and gutless plasmid are

cotransfected into 293 cells. The viruses are then grown together in 293 cells until the final passage through CRE8 cells.

FIG. 8B. Enrichment by complementation. An adenovirus gene is inserted between the lox511 sites in the gutless virus, E4 for example. A helper virus which is an E4 deleted version of Ψ5 would then be cotransected into 293 cells. There should be no sequence in common flanking the E4 gene and the E4 deletion to minimize recombination between helper and gutless viruses. Only those cells containing both viruses will produce virus. The E4 deleted helper virus must be grown on a cell line complementing E1 and E4 genes.

FIG. 8C. A dominant selection in *trans*. A transcriptional regulator protein would be inserted between the lox511 sites in the gutless virus. The helper virus would be modified by placing a selectable marker in the E1 region controlled by the transcriptional regulator. The selection could be either positive or negative. For positive selection, a viral gene would be moved to the E1 region in the helper virus, fiber for example. Under this scenario, fiber gene transcription would require the regulatory protein from the gutless virus. For negative regulation, a poisonous gene such as herpes virus thymidine kinase (tk) would be inserted into the E1 region of the helper virus and gancyclovir added to the growth media. Here the regulatory protein would repress expression of the tk gene; otherwise the combination of tk and gancyclovir would poison viral DNA replication.

FIG. 9. Encapsidated loxβA 'gutless' and Ψ5 helper viruses on 293 and CRE8 cells. Both DNA's were digested with BglII. Two different amounts of each sample is shown.

FIG. 10A and 10B. Titration of a 1::1 mixture of loxβA and Ψ5 on CRE8 cells. Packaged DNA was prepared from 10^7 cells infected at moi's shown. The moi's are approximate and based on a single virus. **FIG. 10A:** The * on the gel marks the position of a 2.2 kb fragment unique to the helper virus. The major band migrating at 2.8 kb is from a pair of loxβA fragments. **FIG. 10B:** the graph shows the result of band intensities of the 2.2 and 2.8 kb bands, corrected for size and molarity.

FIG. 11A and 11B. Effect of DNA phasing on Cre/lox recombination. The Ψ9 and Ψ9+17 viruses were mixed with Ad β-gal virus and used to infect either 293 or CRE8 cells. Packaged DNA was prepared, digested with BsaBI, separated in an agarose gel and the intensities of the labeled bands were determined. **FIG. 11A** is a photograph of a gel showing BsaBI-digested DNAs. **FIG. 11B** is a diagram showing the phasing of the lox sites in the two different viruses, Ψ9 and Ψ9+17.

FIG. 12A and 12B. Relative packaging efficiency of Ψ11 virus. A mixture of 20 parts Ψ11 to one part dl309 was used to infect 10^7 either 293 or CRE8 cells at an moi of 10. Encapsidated DNA was prepared and 1/25 of the DNA was subjected to 10 cycles of PCR with primers which recognized both viral DNA's. There was no product when the viral DNA's were omitted. **FIG. 12A** is a gel showing the results. **FIG. 12B** is a diagram showing the structures of Ad5 and ψ11.

FIG. 13A and 13B. Relative packaging efficiency of Ψ7 virus. Equal numbers of particles of Ψ7 and Ad tet β-gal were mixed and used to infect 10^7 293 or CRE8 cells at an moi of 10 for each virus. **FIG. 13A:** Packaged DNA was digested with BsaBI, separated and the intensities of the left end fragments were determined. **FIG. 13B:** In a measurement of the relative encapsidation efficiency, Ψ7 was encapsidated at 30 and 5.9% of a similar virus with a normal Ad5 packaging site when grown in 293 and CRE8 cells respectively.

FIG. 14. Construction of an E1-substituted adenovirus by using Ψ5 and a replicating vector. Negative pressure on Ψ5 is achieved by intramolecular recombination removing the packaging site in the first step. An intermolecular recombination between the replicating vector and Ψ5 then creates a new virus which has an intact packaging site and carries a recombinant gene, marked Exp. Cassette. The packaging site is labeled Ψ.

DESCRIPTION OF SPECIFIC EMBODIMENTS

The present invention provides an integrated system for the *in vivo* production of substantially pure preparations of packaged adenovirus-based vectors useful for gene therapy and gene transfer and expression in mammalian cells. The

system has several components including a therapeutic recombinant adenovirus vector, a helper recombinant adenovirus vector and a host cell line which expresses Cre recombinase. The present invention provides each of these components individually as well as a method for production of *in vivo* packaged therapeutic rAd vectors using the system.

In brief, the method of the present invention works as follows. A eukaryotic host cell which is susceptible to adenovirus infection is transfected with a therapeutic rAd vector and a helper rAd vector. The therapeutic rAd vector is replicated and packaged in the transfected host by the replication and packaging system that is supplied by the helper virus. The therapeutic rAd vector contains Ad DNA sequences only from the ITRs and the packaging site, the remainder of the DNA in the therapeutic rAd vector is from non-adenovirus sources. The helper rAd vector is constructed so that the packaging site is flanked by *lox* sites which recombine with great efficiency in the presence of Cre recombinase. The recombination of the *lox* sites results in the excision of the packaging site. The efficiency of the excision of the packaging site in the helper can be improved by using more than one set of *lox* sites flanking the packaging site. The system contains the further safeguard that any homologous recombination between the helper rAd vector and the therapeutic rAd vector (whether Cre-mediated or not) can only result in a helper rAd vector in which the packaging site is still flanked by *lox* sites and thus still vulnerable to excision. The transfection produces a mixture of packaged particles, both therapeutic rAd vector particles and helper rAd vector particles. The packaged viral particles from the transfection are isolated by standard methods and used to infect additional host cells. The infection steps may be repeated a number of times until the titer of the viral particles is produced in sufficient quantities. In the final infection step, host cells capable of expressing the Cre recombinase are used. Cre-expressing host cells may be used in all of the transfection and infection steps as well, however, for infection and transfection steps other than the final one, non-Cre expressing cells are preferred because of the possibility that prolonged selective pressure will result in unwanted deletions in the helper rAd vector.

For ease of description of the rAd vectors of the present invention, reference will be made to the genetic map of adenovirus in Figure 1. The terms "left" and "right" are defined with reference to the structure of adenovirus as it appears in Figure 1. The terms "3'" and "5'" are defined with reference to the upper DNA strand as shown in Figure 1. The rAd vectors of the present invention, including the therapeutic rAd vector and the helper rAd vector, while different in some respects from the wild type adenovirus genetic map in Figure 1, still retain certain adenovirus structural landmarks (e.g. ITRs, packaging site) so that the left/right designation retains the same meaning for the rAd vectors as for wild type Ad. The complete sequence of the wild type Ad5 virus is known and can be found in Chroboczek *et al.* Virology 186:280-285 (1992), GENBANK Accession No. M73260.

The therapeutic rAd vectors of the present invention comprise DNA molecules containing adenovirus inverted terminal repeats (ITRs), an adenovirus packaging site and one or more *lox* sites. The therapeutic rAd vectors additionally comprise a foreign DNA sequence of interest. The ITRs and the packaging site are required *in cis* for replication and packaging of the therapeutic vectors *in vivo*. Adenovirus ITRs useful for the therapeutic rAd vectors of the present invention can be the ITRs from any adenovirus as long as they are recognized by the replication and packaging proteins supplied *in trans* by the helper rAd vector. All naturally occurring adenoviruses have inverted terminal repeats although the length and sequence of the ITRs vary among different adenovirus serotypes. The sequences of many of the ITRs are known (see for example, Sussenbach, J. in "The Adenoviruses" pages 35-113 Ginsberg, H. ed. Plenum Press 1984). Preferably, the ITRs in the therapeutic rAd vectors are the ITRs from Ad2, Ad3, Ad4, Ad5, Ad7, Ad9, Ad10, Ad12, Ad18 or Ad31. More preferably the ITRs are from Ad5. The ITRs are oriented in the therapeutic rAd vectors in the same manner in which they are oriented in the naturally occurring adenovirus, that is, the sequences are inverted with respect to one another and occur at the terminals of the therapeutic rAd vectors. In the therapeutic rAd vectors, the ITRs are separated by the *lox* site or sites, the packaging site, and the foreign DNA sequence of interest.

The adenovirus packaging site is required *in cis* for packaging of the DNA into the adenovirus virions. All adenovirus strains analyzed to date contain a packaging site, typically located at the left end of the viral genome, adjacent to and to the right of the left ITR. In addition to the naturally-occurring Ad packaging sites, certain other DNA sequences have been shown empirically to function as packaging sites. Such sequences are referred to as synthetic packaging sites. For the therapeutic rAd vectors of the present invention, any naturally-occurring or synthetic adenovirus packaging site is suitable as long as the site is recognized by the packaging system of the helper rAd virus used. Preferably, the packaging site is that from Ad5. In particular, the Ad5 packaging site is the DNA sequence from base pair 194 to base pair 452 of the Ad5 genome as measured from the left end. More preferably, the packaging site is the DNA sequence from base pair 194 to base pair 375 of the Ad5 genome as measured from the left end. Alternatively, the packaging site useful for the therapeutic rAd vectors of the present invention may be a synthetic packaging site. For example, one synthetic packaging site composed of six tandemly repeated copies of the "A" repeat has been shown the function as a packaging site *in vivo* (Grable and Hearing J. Virol. 64:2047-2056). Preferably, for the therapeutic rAd vectors of the present invention, the packaging site is a naturally-occurring adenovirus packaging site.

The therapeutic rAd vectors of the present invention contain at least one *lox* site for recombination. *Lox* sites are the sites at which Cre-mediated recombination occurs. The presence of at least one *lox* site in the therapeutic rAd vector of the same type as at least one of the *lox* sites in the helper rAd vector serves to insure that any homologous recombination between the therapeutic rAd vector and the helper rAd vector (through the ITRs or packaging sites, for example) can only result in helper rAd vectors which still retain *lox* sites flanking the packaging site. Several types of *lox* sites have been identified and their sequences are well known. Any *lox* site that is capable of undergoing Cre-mediated recombination is suitable for use in the therapeutic rAd vector. Preferably for the therapeutic rAd vectors of the present invention, one or more of the following *lox* sites are useful: *lox* P, *lox* 511, *lox* 514 and *lox* Psym (Hoess *et al*. Nucl. Acids Res. 14:2287-2301 1986). The sequence of several *lox* sites are

shown in Figure 2. The therapeutic rAd vectors of the present invention may contain one or more types of *lox* sites but will contain no more than one *lox* site of each type. At least one of the *lox* site(s) used in the therapeutic rAD vector will be of the same type as at least one of the *lox* sites used in the helper rAd vector as described below.

The therapeutic rAd vectors of the present invention contain a foreign DNA sequence of interest. The foreign DNA sequence of interest typically comprises genes or other DNA sequences that are of interest to transfer into mammalian cells. Foreign DNA sequence of interest may include any naturally occurring or synthetic DNA sequence. The foreign DNA may encode protein, or contain regulatory sites, including but not limited to, transcription factor binding sites, promoters, enhancers, silencers, ribosome binding sequences, recombination sites, origins of replication, sequences which regulate RNA stability and polyadenylation signals. The foreign DNA may be identical in sequence to naturally-occurring DNA or may be mutated relative to the naturally occurring sequence. The foreign DNA need not be characterized as to sequence or function.

The size of foreign DNA that may be included in the therapeutic rAd vector will depend upon the size of the rest of the vector. Preferably, the total size of foreign DNA is from 36 kb to 37.4 kb. The total size of the therapeutic rAd vector will be not larger than about 38 kb. Preferably, the total size of the therapeutic rAd vector is from 32 to 38 kb; more preferably, from 34 kb to 37 kb; and most preferably from 35 kb to 36 kb. The lower size limit for packaging efficiency (i.e. at least 80% of wild type packaging efficiency) is around 32 kb total rAd vector. Recombinant Ad viruses that are smaller than 32 kb are not as stable, which can create significant problems for reliable delivery of the same recombinant vector for gene therapy. This discovery of the optimum packaging size is also important for manufacturing large quantities of the viral particles for commercial use, in which case packaging efficiencies on the order of wild type efficiencies are desired.

The construction of the therapeutic rAd vector of the present invention is accomplished by operationally joining the various required DNA sequences that comprise the therapeutic rAd vector, that is, the left and right ITR sequences, the

adenovirus packaging site sequence, the *lox* site sequence(s) and the foreign DNA sequence of interest. The various required DNA sequences are joined together in a particular order and in a specific orientation with regard to one another. The order and orientation of the sequences is illustrated from the left to right direction as follows: a left inverted terminal repeat, one or more *lox* recombination sites, an adenovirus packaging site, a foreign DNA sequence of interest, a right inverted terminal repeat. The required sequences may be joined directly to one another or additional DNA sequences may intervene between the required sequences. The additional intervening DNA sequences may be residual from the cloning process used to join the required sequences or may be particular sequences positioned between or within the required DNA sequences in order to aid in manipulation or efficiency of the vectors, for example, restriction sites, PCR priming sites, promoters, selectable marker genes and the like. The left and right inverted terminal repeats are oriented toward one another in the therapeutic rAd vector in the same manner as that in which they are found naturally-occurring in the adenovirus, that is they are inversely oriented with respect to one another. By inversely oriented is meant that when read on the same strand in the 5′ to 3′ direction, the ITR sequences are the reverse complements of one another. The orientation of the packaging site is not critical for the vectors of the present invention so that the packaging site may be oriented in either direction with respect to the left ITR. The packaging site will be positioned no more than 400 bp from the left end of the therapeutic rAd vector, preferably no more than 300 bp from the left end of the vector. The *lox* site is oriented with respect to the packaging site in the same manner as are the identical *lox* sites in the helper rAd with respect to the packaging site, as described below. If more than one type of *lox* site is present, the *lox* sites may preferably be ordered in the same manner as they occur on the left side of the packaging site in the particular helper rAd vector to be used with the therapeutic rAd vector. The foreign DNA of interest may be oriented in any appropriate orientation with respect to the rest of the vector. The sequences are joined together by any of a number of techniques that are well known in the DNA cloning art. The joining is most conveniently accomplished by ligation of DNA fragments, for instance restriction fragments or chemically or

enzymatically synthesized DNA fragments, containing the various required DNA sequences. Alternatively, the various required DNA sequences may be chemically or enzymatically synthesized as a single DNA fragment or two or more of the various required sequences may be synthesized as a single fragment and joined to the remaining sequences.

As an alternative to *in vitro* vector construction methods described above, the therapeutic rAd may be constructed *in vivo* by homologous recombination techniques that are well known in the art (see for example, Chinniduri *et al.* J. Virol. 32:623-628 (1979); Bett *et al.* (1994)). *In vivo* construction using Cre-mediated recombination of *lox* sites may also be used to create the therapeutic rAd vector. For this purpose, a precursor therapeutic rAd vector may be constructed without ITR sequences and containing an additional *lox* site, located at the right-most end of the foreign DNA. The precursor therapeutic rAd vector thus has the following sequences in order: a first *lox* site, an Ad packaging site, the foreign DNA, and a second *lox* site not identical to the first site. A modified helper rAd vector is constructed as described below except that an additional *lox* site, identical to the second *lox* site in the precursor therapeutic rAd vector, is positioned adjacent to and to the left of the right ITR. The additional *lox* site is not identical to any of the other *lox* sites in the modified helper. The precursor therapeutic rAd vector and the modified helper rAd are transfected into a Cre-expressing host cell and Cre-mediated recombination results in a transfer of the ITRs from the modified helper to the precursor therapeutic rAd to form a therapeutic rAd vector.

The therapeutic rAd vector may be conveniently cloned into a prokaryotic cloning vehicle (for example, plasmids, bacteriophages, phagemids, etc.) for easy propagation in a bacterial host. Alternatively, a eukaryotic cloning vehicle may be used. For this purpose, restriction sites may be added to the outer most ends of the ITRs. Prior to use for transfection, the therapeutic rAd vector is removed from the cloning vehicle by appropriate restriction digestion. It will be apparent that the therapeutic rAd vector may be constructed first and then cloned into a cloning vehicle or each sequence required in the therapeutic rAd vector may be added separately to the cloning vehicle. Methods of vector construction are well known in the art and one of ordinary skill would readily be able to determine

an appropriate cloning strategy for construction of the therapeutic rAd of the present invention either with or without a cloning vehicle.

The helper rAd vector of the present invention comprises a DNA molecule containing adenovirus genes which encode proteins necessary for the replication and packaging of the therapeutic rAd vectors into therapeutic rAd virus particles. The helper rAd vector of the present invention additionally comprises an adenovirus packaging site, flanked by at least one set of two identical *lox* sites in direct orientation with respect to one another, and a left and a right ITR. The helper rAd vector may contain an entire adenovirus genome provided that any packaging site is flanked by at least one set of two directly-oriented, identical *lox* sites. Particular adenovirus genes may be omitted from the helper rAd virus if the products of the omitted genes are not essential for the replication and packaging of the therapeutic rAd (for example, the E3 proteins, E4 or 1-4 or VAII) or can be supplied otherwise than from the helper rAd vector, for example, from the host cell. The determination of which Ad genes may be omitted from the helper rAd is well within the ability of one of ordinary skill in the art. Preferably, the helper rAd vector will contain the entire adenovirus genome except for the E1a and E1b regions. When the helper rAd vector does not contain the E1a and E1b regions, it is preferably used in combination with a host cell in which the E1a and E1b gene products are supplied from the host. The E1a and E1b gene products are preferably transcribed from heterologous promoters in the host cell.

The adenovirus packaging site suitable for the helper rAd vector of the present invention includes any of the packaging sites that are useful for the therapeutic rAd vector. Preferably, a synthetic packaging site is useful for the helper rAd vector. In general, Ad vectors containing the synthetic packaging sites are less efficiently packaged. Use of such a site therefore contributes to the selection against the helper rAd. Most preferably, the packaging site comprises a DNA sequence of six tandemly repeated "A" repeats (Grable and Hearing, J. Virol. 64:2047-2056).

The packaging site of the helper rAd vector is flanked by at least one set of two identical *lox* sites in direct orientation with respect to one another, one of the set being on the left (5'-most) side of the packaging site and one of the set being

on the right (3'-most) side of the packaging site. By direct orientation is meant that the sequences of the *lox* sites when read in the 5' to 3' direction on the same DNA strand are identical. When more than one set of two identical *lox* sites is present in the helper rAd vector they will be arranged so as to form a nested set; that is, one member of each set will be positioned on the left side of the packaging site and one member will be positioned on the right side of the packaging site and the order of the sites as they occur on the right side of the packaging site will be opposite to that in which they occur on the left side but the two identical members of each set will be in the direct orientation with respect to one another.

One such nested set may be illustrated by a simple example. A helper rAd having three sets of two identical *lox* sites, for instance one set each of *lox* P, *lox* 511 and *lox* 514, may be arranged in order ....*lox* P(→)-*lox* 511(→)-*lox* 514(→)-packaging site-*lox* 514(→)-*lox* 511(→)-*lox* P(→).... where the arrows indicate the directionality of the *lox* sites.

Where more than one set of two identical *lox* sites are used, the identical innermost lox sites will preferably be separated from one another by at least 60 base pairs of spacer DNA, wherein the identical lox sites are in phase with each other (i.e. the distance between the identical lox sites before the recombinase/excision step is a multiple of 10.5 base pairs). The lox sites should also be preferably at least 14 bases apart from the adjacent, non-identical lox sites. The distances between lox sites can be created by addition of spacer DNA. The addition of spacer DNA between the *lox* sites insures that there will be a sufficient amount of intervening DNA between the remaining identical *lox* sites for recombination to occur after recombination has occurred between the innermost *lox* sites in the nested set. The center-to-center distance between the innermost nested lox sites will contain the packaging site, which is greater than the minimal size between lox sites that is required for excision by recombinase--roughly 60 base pairs. The center-to-center distance between the innermost lox sites preferably will be the sum of the size of the packaging site plus additional spacer DNA such that the sum is a multiple of 10.5. The center-to-center distance being a multiple of 10.5 base pairs in length places the lox sites in phase with each other, since there are roughly 10.5 bases per helical turn. The second set of

nested lox sites, flanking the innermost set, should comprise two different lox sites, each preferably at least 14 bases distant from the center of the adjacent, non-identical innermost lox site. The distance between the second set of nested lox sites and the adjacent, non-identical innermost lox sites can be optimized by calculating a distance between the second set of nested lox sites that is a multiple of 10.5 bases pairs after excision of the innermost lox sites, wherein the excision leaves a single innermost lox site and spacer DNA. The phasing of lox sites can also be calculated in designing the third set of flanking lox sites in a nested set lox site configuration.

The spacer DNA inserted between the lox sites may also enhance the Cre mediated recombination. In general, it appears that the spacer sequence may be nonspecific and only its length of importance. For instance, interaction between proteins at two different sites on DNA is maximal when the proteins are in phase, that is on the same face of the DNA helix. There are about 10 base pairs per turn of the DNA helix. Additionally, since the helper virus DNA may be in nucleosomes the phasing of the sites for maximal recombination may reflect nucleosomal phasing of 160 to 200 base pairs. However, some aspects of the sequence in the spacer may also be important. As is well known in the art, certain sequences bend DNA or alter its stiffness. In addition, DNA binding proteins can bend DNA and some DNA binding proteins can displace or phase nucleosomes. The spacer DNA may separate the *lox* sites on the right side of the packaging site or those on the left side of the packaging site or both. Preferably, the spacer DNA will separate the *lox* sites on the right side of the packaging site. As in the therapeutic rAd vector, the packaging site in the helper rAd vector will be no more than 400 base pairs, preferably no more than 300 base pairs, from the left ITR.

The left and right ITRs useful for the helper rAd vector can be the ITRs from any adenovirus as long as they are recognized by replication and packaging proteins expressed by the helper rAd vector. Preferably, the ITRs in the helper rAd vectors are the ITRs from Ad2, Ad3, Ad4, Ad5, Ad7, Ad9, Ad10, Ad12, Ad18 or Ad31. More preferably the ITRs are from Ad5. The ITRs are oriented in the helper rAd vectors in the same manner in which they are oriented in the

naturally occurring adenovirus, that is, the sequences are inverted with respect to one another and occur at the terminals of the helper rAd vectors. In the helper rAd vectors, the ITRs are separated by the packaging site, which itself is flanked by at least one identical set of *lox* sites, and adenoviral DNA which encodes the proteins necessary for replication and packaging.

The helper rAd vector may be constructed using any of a number of cloning techniques well known in the art. Such techniques have been described for the construction of the therapeutic rAd vector and include *in vitro* cloning, *in vivo* recombination, chemical or enzymatic synthesis, and any other appropriate methods.

A Cre-expressing host cell culture for use in the present invention can be made in any of a number of ways that are well known in the art. The Cre expressing host cell may be made by transfection of a mammalian cell culture that is susceptible to infection by adenovirus with a DNA vector containing a functional Cre gene or coding sequence. By functional is meant that the gene or coding sequence also contains those regulatory sequences necessary for transcription, translation and localization in the cell into which it is delivered such that Cre is expressed. For example, Adenovirus susceptible cells may be transfected with a vector carrying the Cre gene from bacteriophage P1, under control of a eukaryotic promoter, for example the immediate-early promoter of CMV. The bacteriophage Cre gene may be modified, using techniques that are well known in the art, to include a Kozak sequence and a nuclear localization signal for maximum translational efficiency and transport into the nucleus. Transfectants may be assayed for Cre activity by any conventional method including Western blots with Cre-specific antibody or functional assays for protein activity (see, for example, Adams *et al.* J. Mol. Biol. 226:661-673). Typically, for use in the present invention, Cre-expressing host cell culture will produce an intracellular Cre concentration of between 0.1μM and 50 μM, preferably between 1μM and 20μM. Preferably, the Cre-expressing host cell culture will also express Ela and E1b and the cell will not contain the adenovirus packaging site.
In addition, the chromosomal or episomal DNA of the Cre-expressing host cell

preferably does not contain any sequences capable of recombination with the helper rAd vector.

The method of the present invention provides for the production of a substantially pure preparation of packaged therapeutic rAd vector particles. By a packaged therapeutic rAd vector particle is meant a therapeutic rAd vector DNA packaged into an adenovirus virion to form an infectious particle. The packaged therapeutic rAd vector particles may also be referred to as therapeutic rAd virus. By infectious is meant that the packaged particle is at least capable of binding to the high affinity Ad receptor on the host cell, followed by internalization and transport of the DNA to the nucleus. By substantially pure is meant a preparation in which at least 95% of the vector particles present are therapeutic rAd vector particles; preferably at least 99% of the vector particles present are therapeutic rAd vector particles, more preferably at least 99.5% of the vector particles present are therapeutic rAd vector particles.

The method of the present invention is initiated by transfecting a host cell susceptible to adenovirus infection with a therapeutic rAd vector and a helper rAd vector. The host cell preferably does not contain any chromosomal or episomal DNA which is capable of recombination with the helper rAd vector. The host cell used for transfection may be any cell culture susceptible to Adenovirus infection. The host may be one which is capable of expressing Cre recombinase, but non-Cre-expressing host cells are preferred for the transfection step. The choice of a particular therapeutic rAd vector and a particular helper rAd vector is limited only by two requirements: (1) that the replication and packaging proteins encoded by the helper rAd vector must be able to recognize the ITRs of the helper rAd vector and the ITRs and packaging site of the therapeutic rAd vector and (2) that the therapeutic rAd vector must contain at least one *lox* site to the left of the packaging site that is identical to one of the *lox* sites in the equivalent position in the helper rAd vector. If the rAd vectors have been cloned into and propagated in cloning vehicles, the cloning vehicle DNA may be removed prior to transfection such that only the rAd vector is used. The cloning vehicle DNA may be removed in any of a number of ways that are well known in the art, for example by

restriction digestion or by synthesis of PCR fragments containing only the rAd vector sequences.

Transfection may be performed by the DEAE-dextran method (McCutchen and Pagano, 1968, J. Natl. Cancer Inst. 41:351-357), the calcium phosphate procedure (Graham *et al.*, J. Virol. 33:739-748 (1973); Graham and van der Eb, Virology 52:456-467 (1973)) or by any other method known in the art, including but not limited to microinjection, lipofection, and electroporation. In most cases, the cells will be transfected with the helper rAd and the therapeutic rAd simultaneously, although there may be instances in which it is more appropriate to transfect with each vector separately. Typically, an equimolar amount of the helper rAd vector and the therapeutic rAd vector will be used but this may be varied for optimal yields depending on the vectors used. Determination of the appropriate ratio for transfection is well within the skill of one of ordinary skill in the art.

The transfected cells are cultured in a suitable medium, for example DMEM, for a time sufficient to provide for maximum replication and packaging of the therapeutic rAd vectors into viral particles. The point of maximum replication and packaging of the therapeutic rAd can be estimated by determining the viral yield at various times after transfection. The viral yield can be determined by conventional methods, including infection of Ad susceptible host with lysates and counting the number of infected cells expressing a marker gene contained in the foreign DNA, quantitative PCR for sequences unique to the therapeutic rAd, measurement of absorbance of the viral preparation at 280 nm after correcting for any helper virus that might be present (the titer of any contaminating helper rAd virus is easily determined by plaque assays), or by measurement of absorbance of the viral DNA at 260 nm (any contamination with helper rAd DNA can be determined by appropriate restriction digestion). In most cases, 72 hours is a sufficient time period for culturing the transfected cells. Following the transfection and during the culturing period, the helper rAd is transcribed and translated to produce the adenovirus gene products necessary for the replication and packaging of the therapeutic rAd.

The packaged vector particles are isolated from the transfected cells by conventional means. Conventional means includes producing a lysate of the whole plate or overlaying the monolayer of cells with agar and then removing only the infected cells in the plaques. Plaques are made of dead infected cells and are visible to the eye. The plaques may also be stained for expression of a marker gene in the rAd virus. Plaque isolation is typically done at about a week after transfection. The vector particles isolated from the transfected cells are a mixture of packaged therapeutic rAd vector particles and packaged helper rAd vector particles. The mixture of vector particles isolated from the transfected cells is used to infect a fresh host cell culture by methods that are well known in the art. Packaged viral particles are isolated from the infected cells and the infection process is repeated with fresh host cells in order to amplify the viral titer with each passage. Since the yield of rAd virus from the transfection is quite small, it is important to infect a small number of cells (10^2). At each passage the cell number is increased 10^2 to 10^4 to 10^6 to 10^8. Because infection with the packaged vector particles is much more efficient than transfection with the naked vector DNA, more of the cells will receive and replicate the vectors in the infection steps.

As in the transfection, the co-infection yields a mixture of packaged rAd particles. The packaged rAd vector particles are isolated from the infected cells in the same manner as from the transfected cells and may be used to repeat the infection steps as many times as necessary to produce a sufficient titer of substantially pure packaged therapeutic rAd vector particles. In general the titer will be between 10^7 and 10^{10} per ml, preferably between 10^9 and 10^{10} per ml.

In the final step of the method of the present invention, a Cre-expressing cell line is used as the host cell for infection. In the Cre-expressing cells, the helper rAd undergoes replication but the packaging of the helper rAd will be limited because Cre-mediated recombination between the *lox* sites will result in the excision of the packaging site from a large percentage of the helper rAd molecules. Helper rAd without the packaging site is not packaged into virions. When one set of *lox* sites is present in the helper rAd, packaging site will be excised in about 95% of the molecules; when two sets of *lox* sites are used, the

molecules with an excised packaging site increases to about 99%; when three sets are used the percentage increases to about 99.99%. In addition, any host-mediated recombination that may occur between the therapeutic rAd vector and the helper rAd vector (for example, due to the possible homology of the ITRs or the packaging sites) during the replication and packaging process will not result in regeneration of a wild type adenovirus. Because the therapeutic rAd vector contains a *lox* site between the left ITR and the packaging site, any recombination with the helper rAd will result only in a helper vector in which the packaging site is still flanked by at least one set of identical *lox* sites as long as the *lox* site in the therapeutic virus is identical to at least one of the *lox* sites in the helper rAd vector.

Typically the method of the present invention is carried out as follows. The therapeutic rAd vector and the helper rAd vector are transfected into non-Cre-expressing cells (that is, non-selective cells). The monolayer of transfected cells is overlain with agar and plaques are removed after one week. The virus mixture from the plaques is passed successively by infection onto increasing numbers of non-Cre-expressing (non-selective) cells; 10^7 non-Cre-expressing cells are infected and the resulting viral DNA is screened for the presence of the rAd virus. The helper virus is reduced in the final passage by infecting Cre recombinase expressing cells. By limiting passage of the helper rAd through Cre-expressing host cells to the final infection step, helper virus is not under continuous selection and the possibility of mutations rendering the helper resistant to Cre selection is lessened.

In another embodiment, the present invention comprises a method for rapidly and efficiently generating new recombinant adenovirus vectors with substitutions in the E1 region or any other adenoviral region. This method uses a helper rAd of the present invention, for example Ψ5, and Cre-expressing host cells to generate new adenovirus recombinants *in vivo*. The helper vector comprises a packaging site flanked by recombination sites and an ITR, wherein the packaging site can be located at either end of the helper vector. The helper vector can also contain adenoviral or foreign DNA sequences between the recombination sites, wherein the adenoviral or foreign DNA sequences can be excised or deleted via

recombinase-mediated recombination. The adenoviral DNA to be deleted can be from any region of the adenoviral genome expressing genes that can be complemented. One advantage of deleting adenoviral or foreign DNA sequences is to provide room for substitute DNA. The helper rAd vector is transfected or infected into Cre-expressing host cells along with a replicating vector containing the Ad ITRs separated by an Ad packaging site, the substitute DNA to be substituted into the E1 region (or any region in which a substitution is preferred) and a *lox* site identical to at least one of the *lox* sites in the helper rAd vector. During the growth of the virus in the transfected cells, the packaging site in the helper rAd is excised by Cre-mediated recombination to yield a deleted helper rAd (Δ-helper) containing no packaging site and only a single *lox* site. Recombination of the Δ-helper with the replicating vector at the *lox* site yields packageable virus containing the essential viral genes from the Δ-helper and the substitute DNA from the replicating vector. If the substitute DNA includes adenoviral genes that are necessary for replication, then the resulting substituted rAd vector will be a replicating virus. On the other hand, if the substitute DNA does not introduce adenoviral genes that are necessary for replication and are not otherwise available, then the resulting substituted rAd vector will be a nonreplicating virus.

One of ordinary skill in the art will appreciate that this method can be used to generate new rAd vectors with substitutions in any Ad region, including but not limited to the E1 region.

By replicating vector is meant any vector that can be replicated by the adenovirus replication system. The replicating vector must contain Ad inverted terminal repeats. The replicating vector will also contain an Ad packaging site and a *lox* site. Preferably the replicating vector will additionally contain substitute DNA inserted between the packaging site and the *lox* site. The replicating vector is preferably pAd*lox* or pAd*lox* derivatives having the substitute DNA inserted in the polylinker region. By substitute DNA is meant any DNA to be substituted into the E1 or other Ad region.

By using selection against Ψ5, a recombinant adenovirus carrying substitute DNA in place of the E1 genes was generated by cotransfecting a replicating vector with a *loxP* site (pAdlox) and Ψ5 DNA into a CRE8 cells (Fig. 14). In the first

step of the reaction, Cre recombinase catalyzes recombination between the two *loxP* sites in Ψ5, removing the packaging site from the virus. In the second step, Cre recombinase catalyzes a recombination between Ψ5 and pAdlox, transferring the substitute DNA into Ψ5. The resulting recombinant virus will now have a single *loxP* site and therefore will have a considerable growth advantage over Ψ5 in CRE8 cells. This growth advantage should generate virus stocks comprised predominantly of the recombinant adenovirus having a substitution.

Specific examples of the steps described above are set forth in the following examples. However, it will be apparent to one of ordinary skill in the art that many modifications are possible and that the examples are provided for purposes of illustration only and are not limiting of the invention unless so specified.

EXAMPLES

EXAMPLE 1. CONSTRUCTION OF THE Ψ5 HELPER RAD VECTOR

The starting material for construction of the helper rAd vector Ψ5 was a plasmid containing the following sequences in order: an SfiI restriction site, nucleotides 2 to 553 from Ad5 (nucleotide 1 is the last C of the SfiI site), an XhoI restriction site, the human CMV immediate-early promoter from -600 to +1 relative to the start of transcription, the polylinker sequence from pSP73 from the HindIII site to the EcoRI site, a polyadenylation signal from SV40 (nucleotides 2752 to 2534 of SV40), pSP73 sequence from nucleotide 2 through 2382 (containing ClaI, EcoRV, and BglII sites), an ApaI restriction site, the right ITR from Ad5. The ApaI-SfiI fragment containing the right ITR was made by polymerase chain reaction. For ease of manipulation the SfiI fragment was cloned into the PvuII site of pBluescript$^+$ to give pCMV-Ad. Cleavage of pCMV-Ad with SfiI releases the original SfiI fragment.

Next, a single *lox* P site (chemically synthesized) was inserted into pCMV-Ad between the ClaI and BglII sites to give plasmid pAd*lox*. A second *lox* P site was inserted into pAd*lox* between nucleotides 193 and 194 of the Ad5 left end fragment to give pf*lox*Pac. The orientation of the two *lox* P sites in pf*lox*Pac is

WHAT IS CLAIMED IS:

1. A recombinant adenovirus vector comprising:

(a) a left inverted terminal repeat;

(b) one or more different recombination sites;

(c) an adenovirus packaging site;

(d) a foreign DNA sequence; and

(e) a right inverted terminal repeat;

wherein said recombinant adenovirus vector is incapable of expressing any adenovirus genes.

2. The vector of claim 1, wherein said vector contains no more than 600 basepairs of adenovirus sequence.

3. A therapeutic recombinant adenovirus vector comprising:

(a) a foreign DNA sequence, wherein said foreign DNA sequence comprises a sequence encoding a therapeutically effective polypeptide; and

(b) adenovirus DNA consisting essentially of left and right inverted terminal repeats, an adenovirus packaging site, and one or more different recombination sites, wherein said vector ranges in size from 32 kb to 38 kb.

4. The vector of claim 3, wherein said vector ranges in size from 34 to 37 kb.

5. The vector of claim 4, wherein said vector ranges in size from 35 to 36 kb.

6. The vector of claim 3, wherein said foreign DNA sequence ranges in size from 31.4 to 37.4 kb.

7. The vector of claim 3, wherein said adenovirus DNA sequence contains a lox site.

8. A recombinant adenovirus helper vector comprising:

(a) left and right inverted terminal repeats;

(b) one or more adenovirus genes necessary for either replication or packaging of adenovirus vectors; and

(c) a packaging site flanked by at least a first pair of identical recombination sites.

9. The helper vector of claim 8, wherein said identical recombination sites are in phase with each other.

10. The helper vector of claim 8, wherein said first pair of identical recombination sites is flanked by a second pair of identical recombination sites, wherein said recombination sites of said first and second pairs are different.

11. The helper vector of claim 10, wherein said first pair of identical recombination sites are loxP sites and said second pair of identical recombination sites are lox511 sites.

12. The helper vector of claim 10, further comprising a third set of identical recombination sites different from said first and second sets, wherein said second set is flanked by said third set.

13. The helper vector of claim 10, wherein said identical recombination sites are in phase with each other.

14. The helper vector of claim 8, wherein said packaging site is a synthetic packaging site.

15. The helper vector of claim 8, wherein said packaging site is from an adenovirus serotype different from the adenovirus serotype from which the one or more adenovirus genes are derived.

16. The helper vector of claim 8, wherein said packaging site is no more than 600 base pairs from the left ITR or from the right ITR.

17. A method of preparing a therapeutic recombinant adenovirus comprising:

(a) obtaining a therapeutic recombinant adenovirus vector comprising a foreign DNA sequence and adenovirus DNA sequences consisting essentially of left and right inverted terminal repeats, an adenovirus packaging site, and one or more different recombination sites;

(b) obtaining a recombinant adenovirus helper vector comprising left and right inverted terminal repeats, one or more adenovirus genes encoding proteins necessary for either replication or packaging of adenovirus vectors, and a packaging site flanked by at least a first pair of identical recombination sites in direct orientation with respect to one another, wherein for each pair of identical recombination sites present in said helper vector there is one identical recombination site in said therapeutic recombinant adenovirus vector,

(c) transfecting a eukaryotic host cell susceptible to adenovirus infection with said therapeutic recombinant adenovirus vector and said recombinant adenovirus helper vector;

(d) isolating packaged viral particles; and

(e) infecting a eukaryotic host cell with said isolated packaged viral particles, wherein said eukaryotic host cell is susceptible to adenovirus infection and is capable of expressing a recombinase capable of mediating recombination between said recombination sites.

18. The method of claim 17, wherein the polypeptides encoded by said helper vector are capable of recognizing the ITRs of the helper vector and the ITRs and packaging site of the therapeutic rAd vector.

19. The method of claim 17, wherein said recombination sites are loxP sites and said recombinase is Cre.

20. The method of claim 17, wherein said host cell is capable of expressing Cre in an intracellular concentration of between 0.01 μM and 50 μM.

21. The method of claim 17, wherein steps (d) and (e) are repeated once.

22. The method of claim 17, wherein steps (d) and (e) are repeated twice.

23. A system for preparing therapeutic recombinant adenovirus vector particles comprising:

(a) a therapeutic recombinant adenovirus vector comprising a foreign DNA sequence, wherein said foreign DNA sequence comprises a sequence encoding a therapeutically effective polypeptide, and adenovirus DNA consisting essentially of left and right inverted terminal repeats, an adenovirus packaging site, and one or more different recombination sites, wherein said vector ranges in size from 32 kb to 38 kb;

(b) a recombinant adenovirus helper vector comprising left and right inverted terminal repeats, and a packaging site flanked by at least a first pair of identical recombination sites;

(c) a eukaryotic host cell susceptible to adenovirus infection, wherein said host cell is capable of expressing of a recombinase capable of mediating recombination between said recombination sites;

wherein for each pair of identical recombination sites in said helper vector there is an identical recombination site in said therapeutic recombinant adenovirus vector.

24. The system of claim 23, wherein said first pair of identical recombination sites in said helper vector are flanked by a second pair of identical recombination sites, and said recombination sites of said first and second pairs are different.

25. The system of claim 24, wherein one pair of recombination sites are loxP and another pair of recombination sites are lox511.

26. The system of claim 23, wherein said foreign DNA sequence ranges in size from 36 kb to 37.4 kb.

27. The system of claim 23, wherein at least one pair of recombination sites are in phase with each other.

28. A substantially pure preparation of packaged therapeutic recombinant adenovirus vector particles, wherein said particles are prepared by the method of claim 16.

29. A composition for treating a genetic disorder comprising a therapeutically effective amount of a substantially pure preparation of packaged therapeutic recombinant adenovirus vector particles, wherein said particles comprise the vector of claim 1.

30. A method of treating a mammal having a genetic disorder, comprising administering the composition of claim 29.

31. A method of generating new recombinant adenovirus vectors with substitutions comprising:

(a) providing a helper vector of claim 8,

(b) providing a replicating vector comprising an adenovirus packaging site flanked by adenovirus ITRs, a recombination site, substitute DNA inserted between said packaging site and said recombination site, wherein said recombination site is identical to said first pair of recombination sites in said helper vector;

(c) transfecting recombinase-expressing host cells with said helper vector and said replicating vector;

(d) isolating packaged viral particles; and

(e) infecting recombinase-expressing host cells with said isolated packaged viral particles.

32. A method of generating new recombinant adenovirus vectors with substitutions comprising:

(a) providing a helper vector of claim 8,

(b) providing a non-replicating vector comprising an adenovirus ITR, an adenovirus packaging site, a substitute DNA sequence, and a recombination site,

wherein said recombination site is identical to said first pair of recombination sites in said helper vector;

(c) transfecting recombinase-expressing host cells with said helper vector and said replicating vector;

(d) isolating packaged viral particles; and

(e) infecting recombinase-expressing host cells with said isolated packaged viral particles.

33. A method of generating new recombinant adenovirus vectors with substitutions comprising:

(a) providing a helper vector of claim 8,

(b) providing a replicating vector comprising an adenovirus packaging site flanked by adenovirus ITRs, a recombination site, substitute DNA inserted between said packaging site and said recombination site, wherein said recombination site is identical to said first pair of recombination sites in said helper vector;

(c) transfecting recombinase-expressing host cells with said replicating vector and infecting with a helper virus derived from said helper vector;

(d) isolating packaged viral particles; and

(e) infecting recombinase-expressing host cells with said isolated packaged viral particles.

34. The method of claim 31, wherein said substitutions are in the E1 region, said recombinase-expressing host cells are cre-expressing host cells comprising E1A+ and E1B+ adenovirus genes, and said recombination sites are lox sites.

35. A system for generating new recombinant adenovirus vectors with substitutions comprising:

(a) a helper vector of claim 8,

(b) a replicating vector comprising an adenovirus packaging site flanked by adenovirus ITRs, a recombination site, substitute DNA inserted between said

packaging site and said recombination site, wherein said recombination site is identical to said first pair of recombination sites in said helper vector; and

(c) a recombinase-expressing host cell line susceptible to adenovirus infection.

5

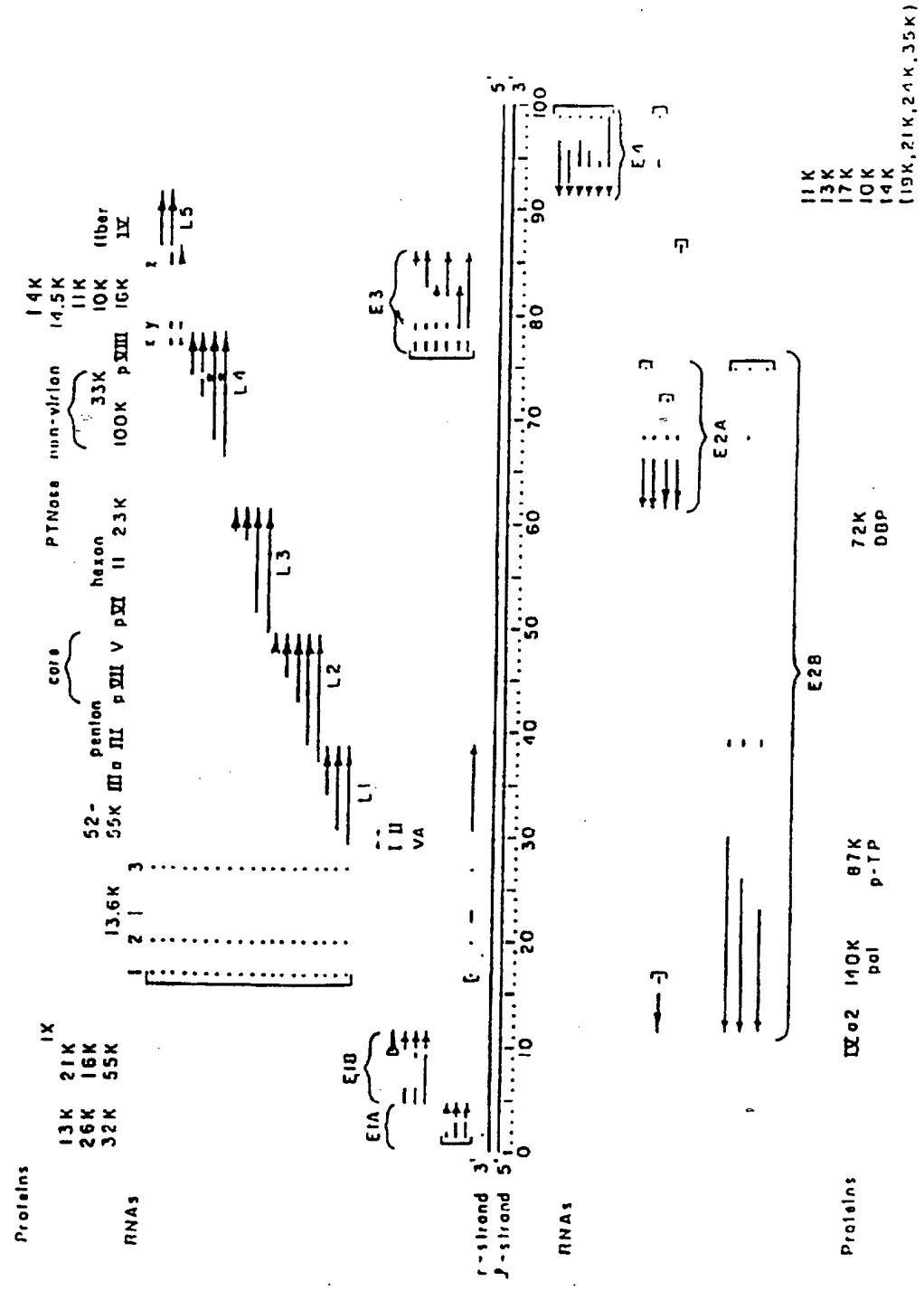

Proteins
IX
13K 21K
26K 16K
32K 55K
RNAs
52-55K
penton
III a IIIa
core
pVII V pVI
hexon
II 23K
PTNase
non-virion
33K
100K pVIII
fiber
14K 14.5K
11K 10K
IV 16K
L1
L2
L3
L4
L5
E1A
E1B
E2A
E2B
E3
E4
IX a2
140K
pol
87K
p-TP
72K
DBP
r-strand 3'
l-strand 5'
0 10 20 30 40 50 60 70 80 90 100
11K
13K
17K
10K
14K
(19K,21K,21K,35K)

FIGURE 1

LOX SITES

lox P	(SEQ ID NO:1)	ATAACTTCGTATA ATGTATGC TATACGAAGTTAT
lox 511	(SEQ ID NO:2)	ATAACTTCGTATA ATGTATAC TATACGAAGTTAT
lox 514	(SEQ ID NO:3)	ATAACTTCGTATA ATGTACGC TATACGAAGTTAT
lox Psym	(SEQ ID NO:4)	ATAACTTCGTATA ATGTACAT TATACGAAGTTAT

FIGURE 2

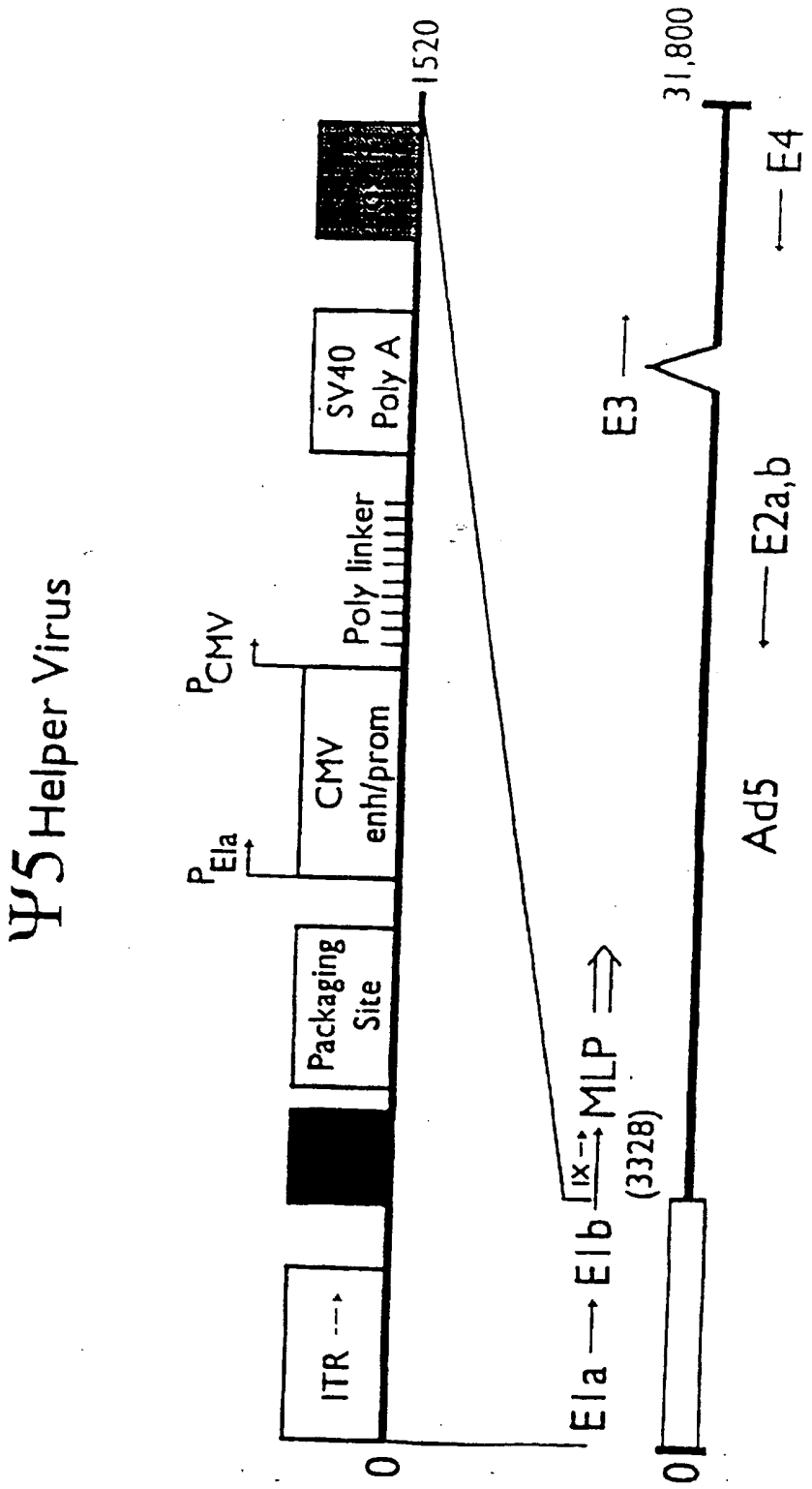

Ψ5 Helper Virus
ITR
Packaging Site
CMV enh/prom
P_{E1a}
P_{CMV}
Poly linker
SV40 Poly A
1520
0
E1a
E1b
IX
MLP
(3328)
E3
E4
E2a,b
Ad5
31,800
0

FIGURE 3

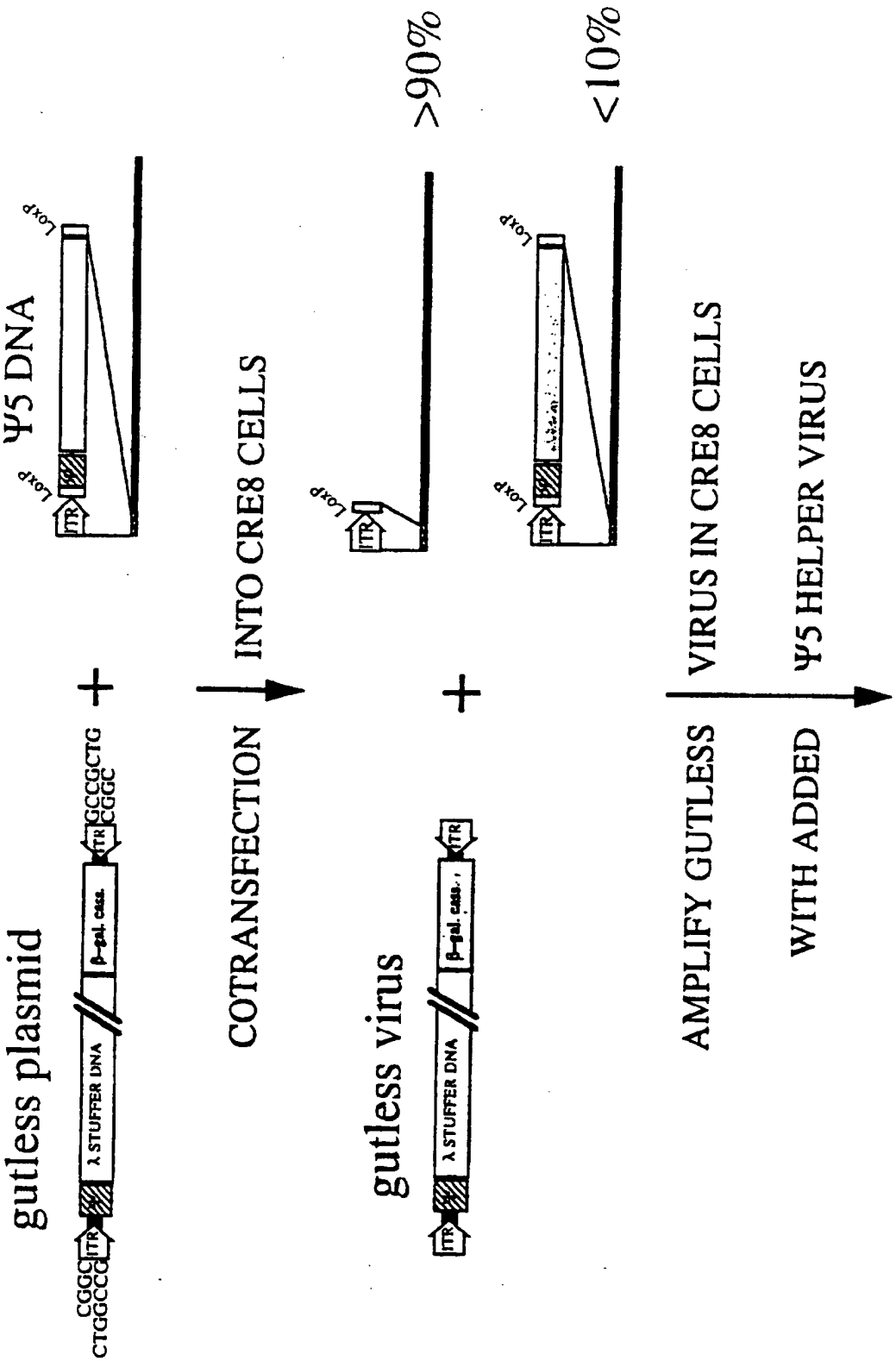

gutless plasmid
CGGC
CTGGCCG
ITR
λ STUFFER DNA
β-gal. cass.
ITR
GCCGCTG
CGGC
+
COTRANSFECTION
INTO CRE8 CELLS
Ψ5 DNA
LOXP
LOXP
ITR
gutless virus
ITR
λ STUFFER DNA
β-gal. cass.
ITR
+
>90%
<10%
AMPLIFY GUTLESS
VIRUS IN CRE8 CELLS
WITH ADDED
Ψ5 HELPER VIRUS

FIG. 4.

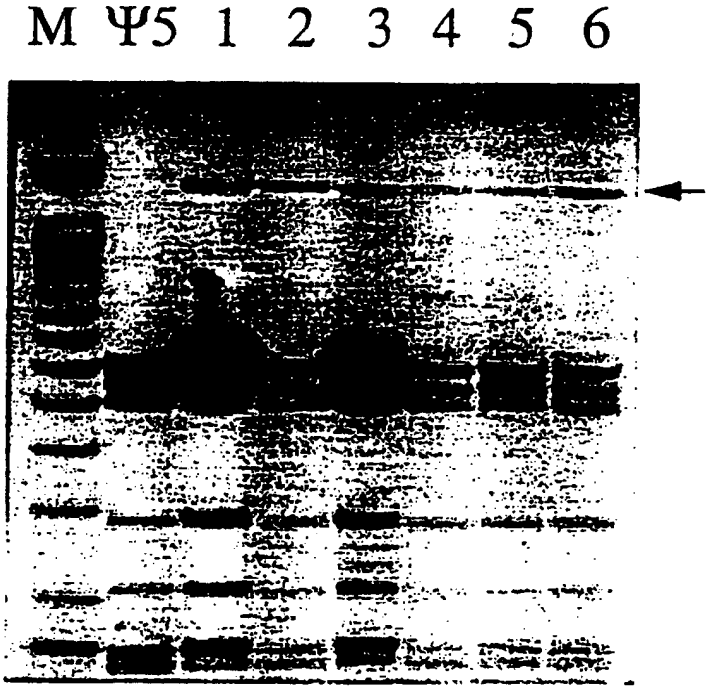


FIG. 5.

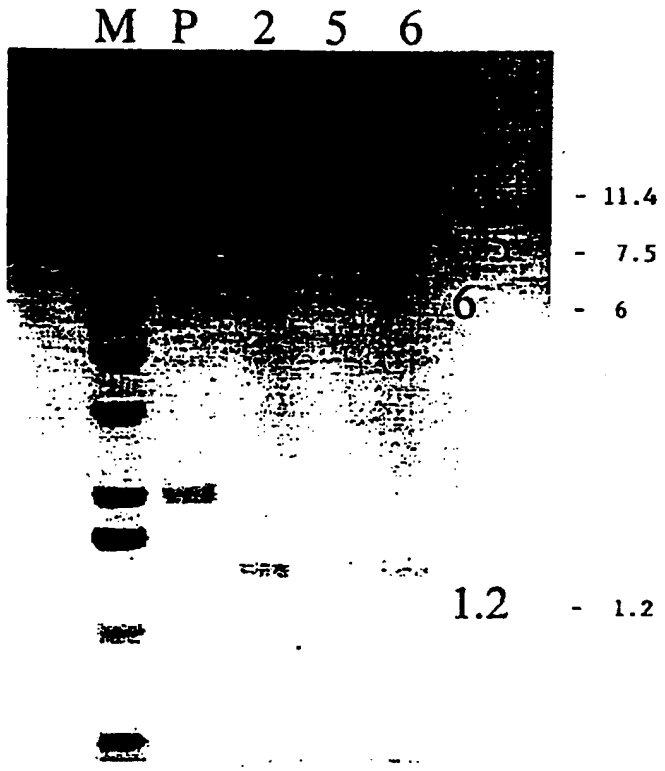


FIG. 6.

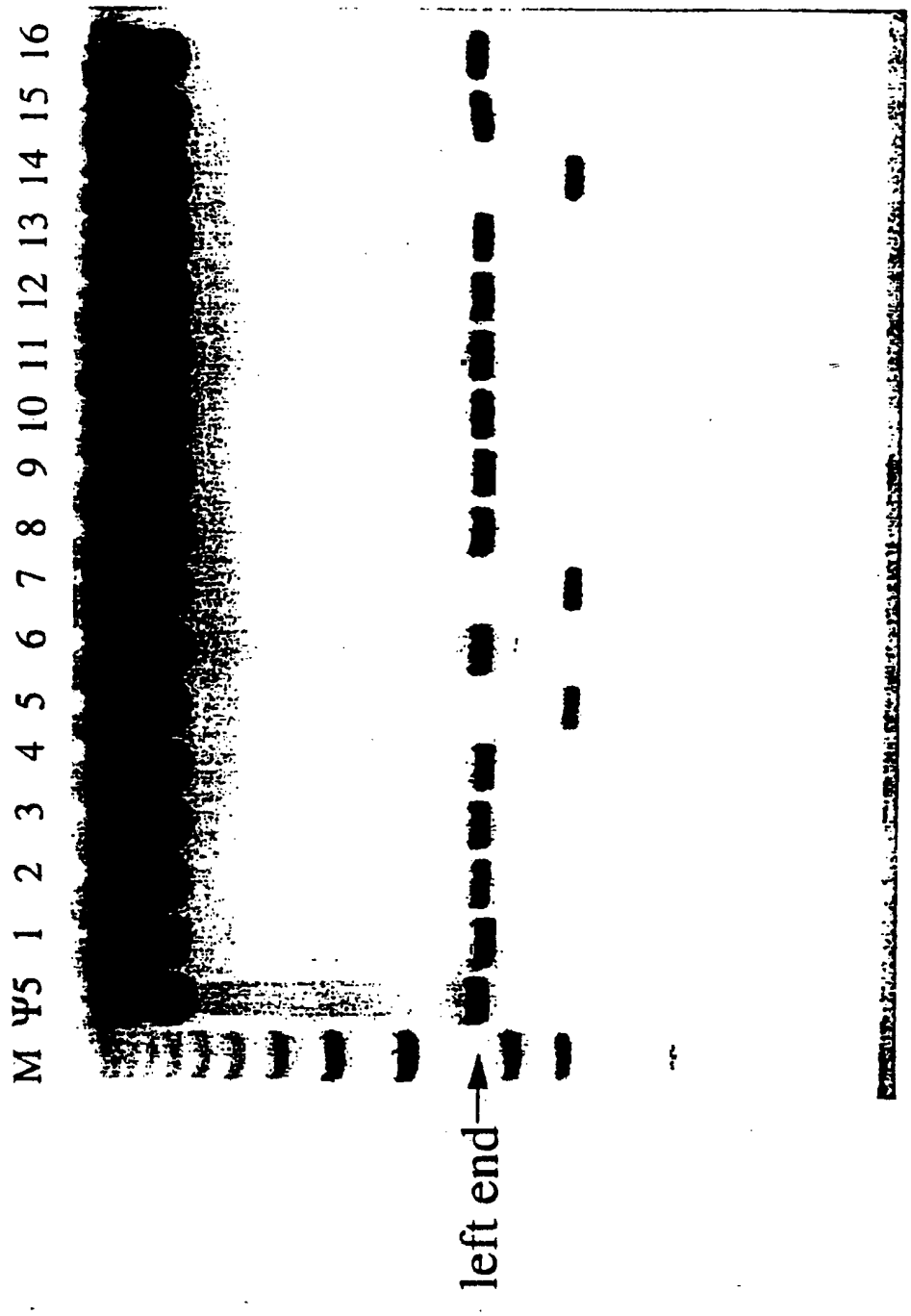
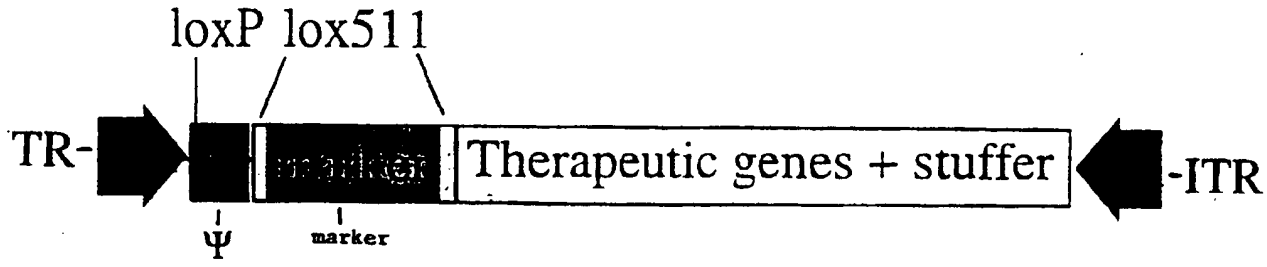


FIG. 7.

Selections for gutless virus.



A) Cell sorting by detection of a passive marker.

marker genes: lacZ, AP, GFP, CD24, truncated NGFR

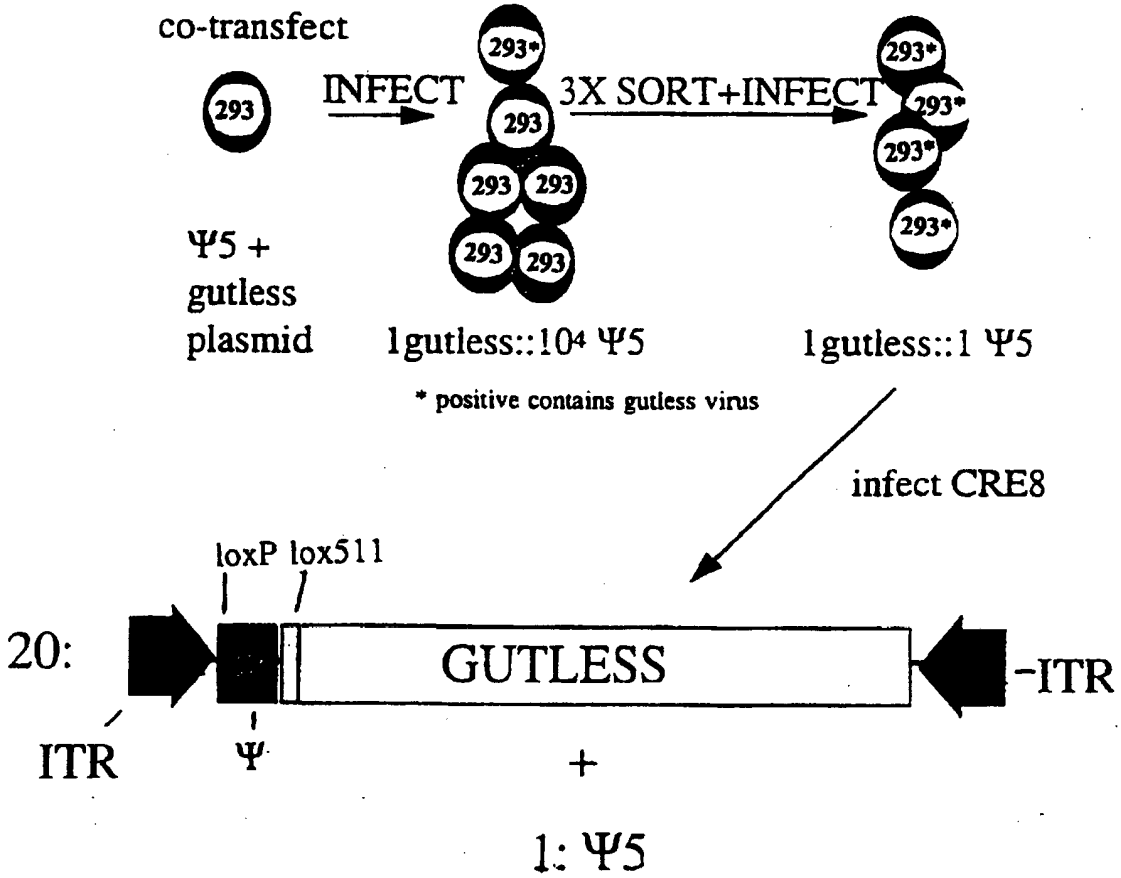


FIG. 8A.

B) COMPLEMENTATION OF A VIRAL FUNCTION SUCH AS E4 OR VAI

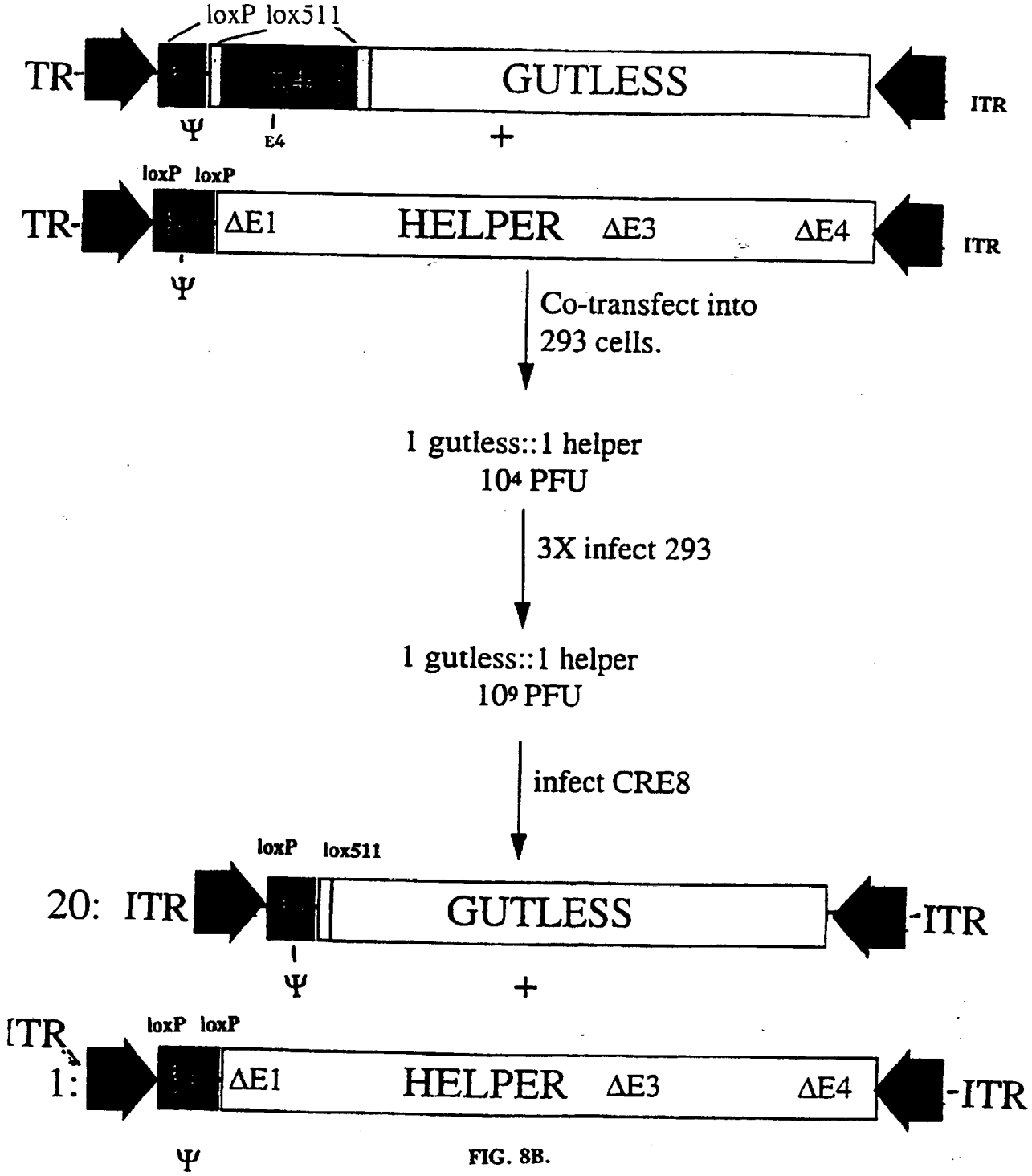


FIG. 8B.

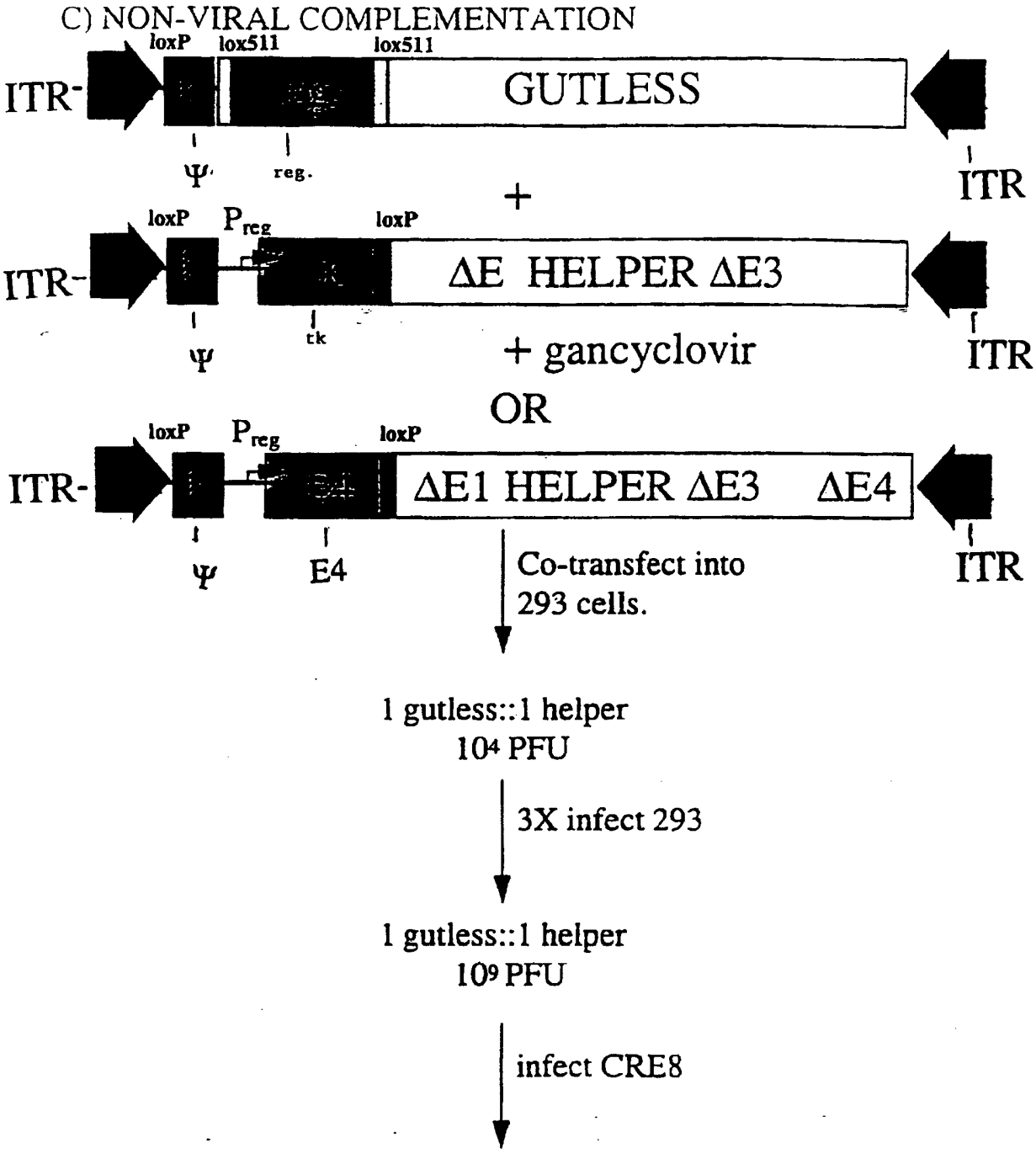

C) NON-VIRAL COMPLEMENTATION
loxP
lox511
lox511
ITR-
GUTLESS
Ψ
reg.
ITR
+
loxP
Preg
loxP
ITR-
ΔE HELPER ΔE3
Ψ
tk
ITR
+ gancyclovir
OR
loxP
Preg
loxP
ITR-
ΔE1 HELPER ΔE3 ΔE4
Ψ
E4
ITR
Co-transfect into
293 cells.
1 gutless::1 helper
10⁴ PFU
3X infect 293
1 gutless::1 helper
10⁹ PFU
infect CRE8
20 GUTLESS:: 1 HELPER

FIG. 8C.

GUTLESS VIRUS ENRICHMENT BY GROWTH IN CRE8 CELLS

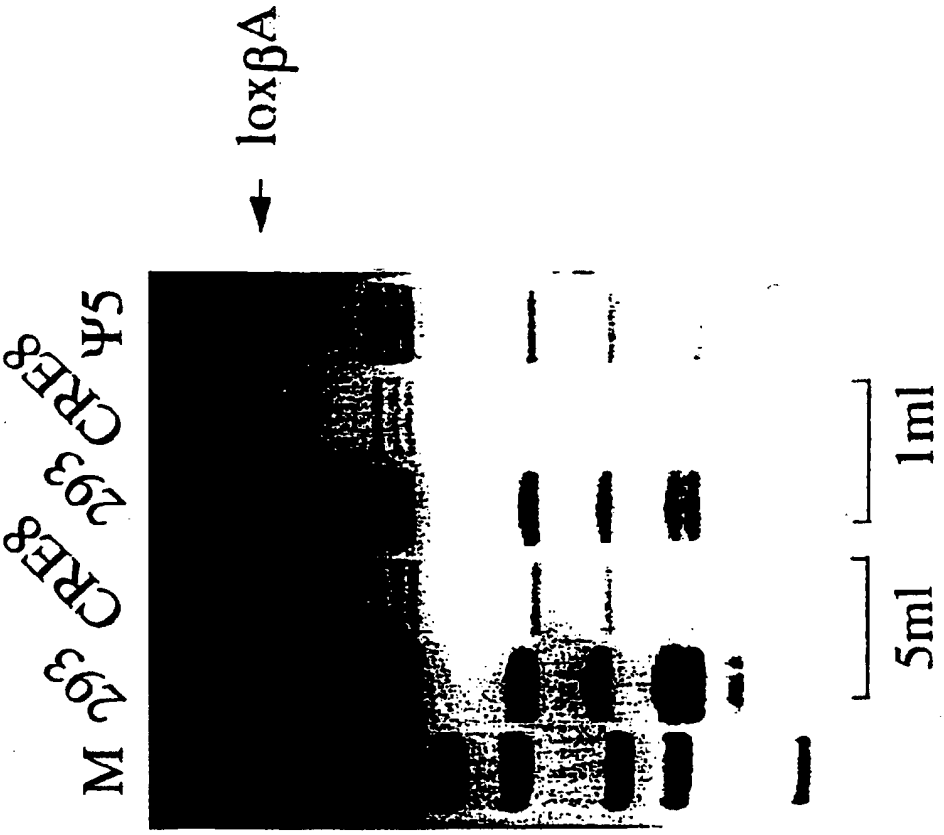
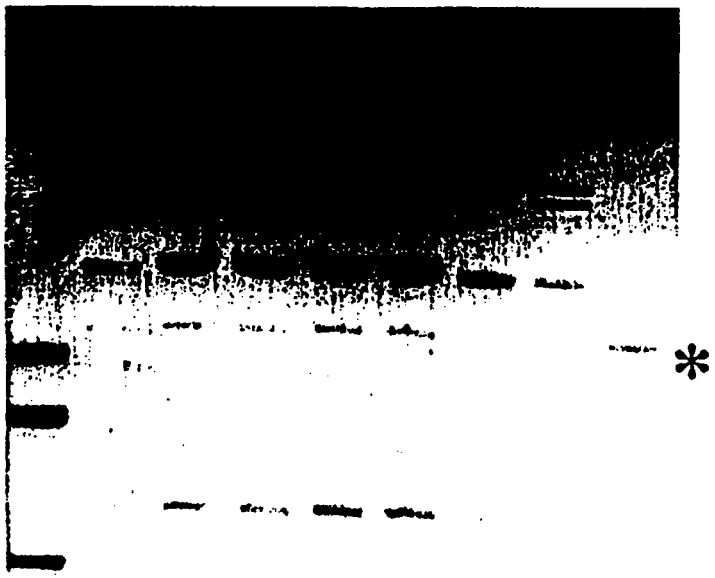


FIG. 9.

TITRATION OF GUTLESS + HELPER VIRUSES (1::1) ON CRE8 CELLS

10A.



10B.

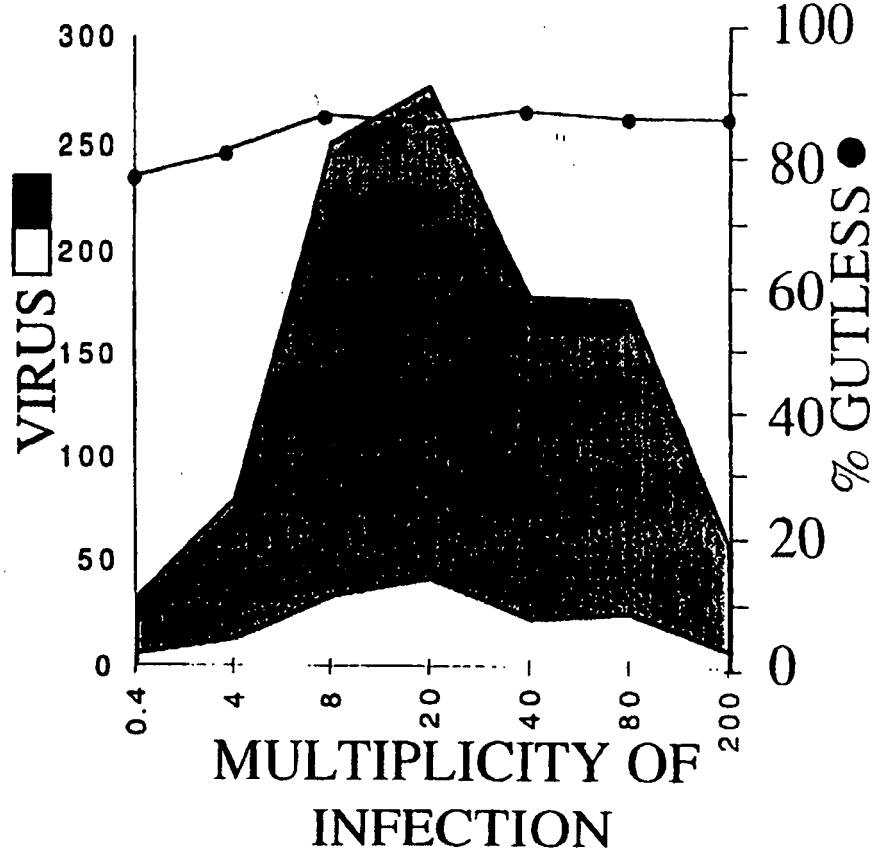


FIG. 10.

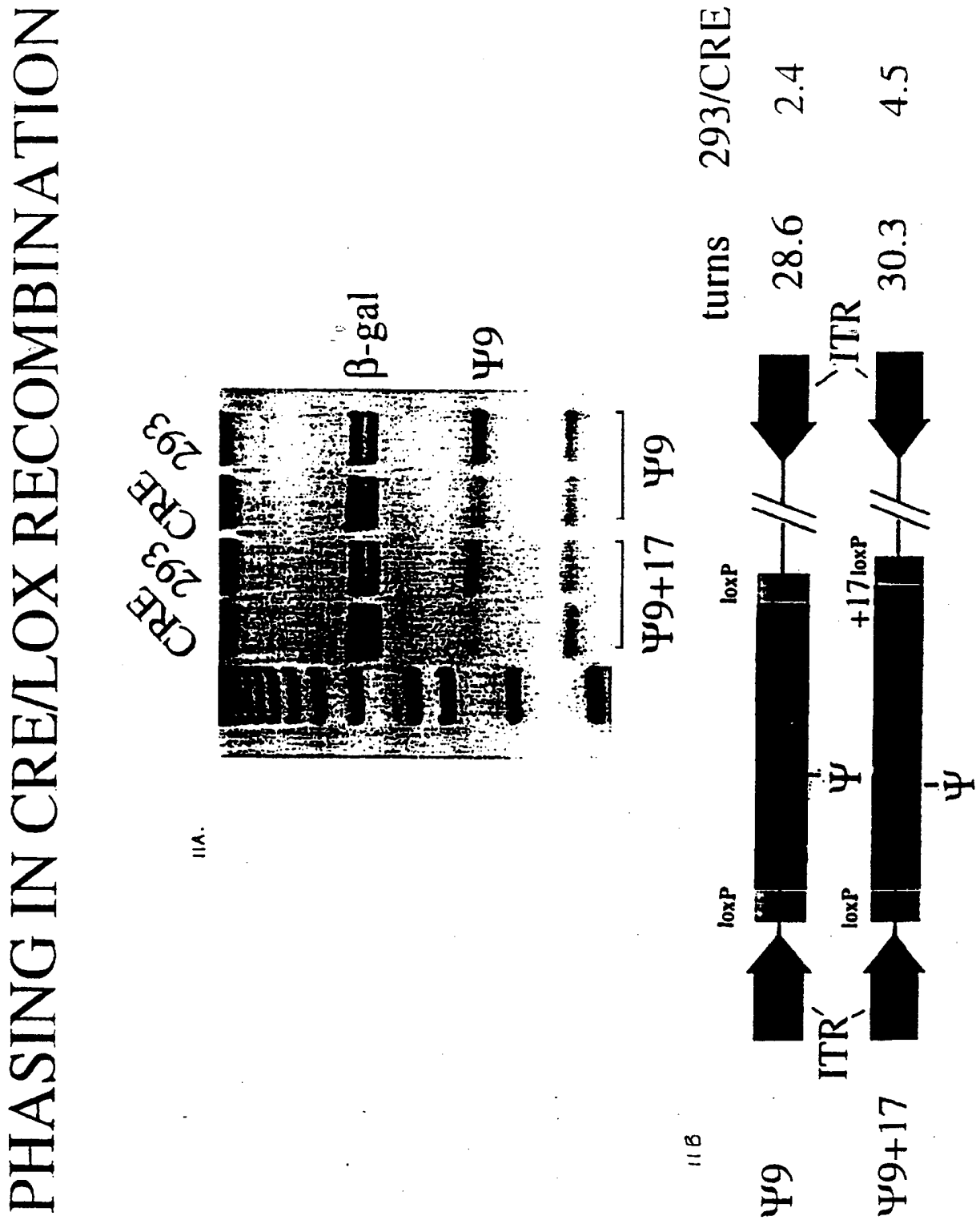
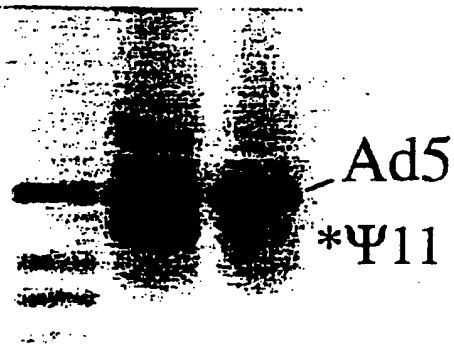

PHASING IN CRE/LOX RECOMBINATION
293
293/CRE
β-gal
Ψ9
Ψ9+17
Ψ9
11A.
293/CRE
turns
28.6
2.4
30.3
4.5
ITR
loxP
+17loxP
Ψ
Ψ
ITR
loxP
loxP
Ψ9
Ψ9+17
11B

FIG. 11.

Ψ11 PACKAGING IN 293 AND CRE8 CELLS

12A.



M 293 CRE8

12B.

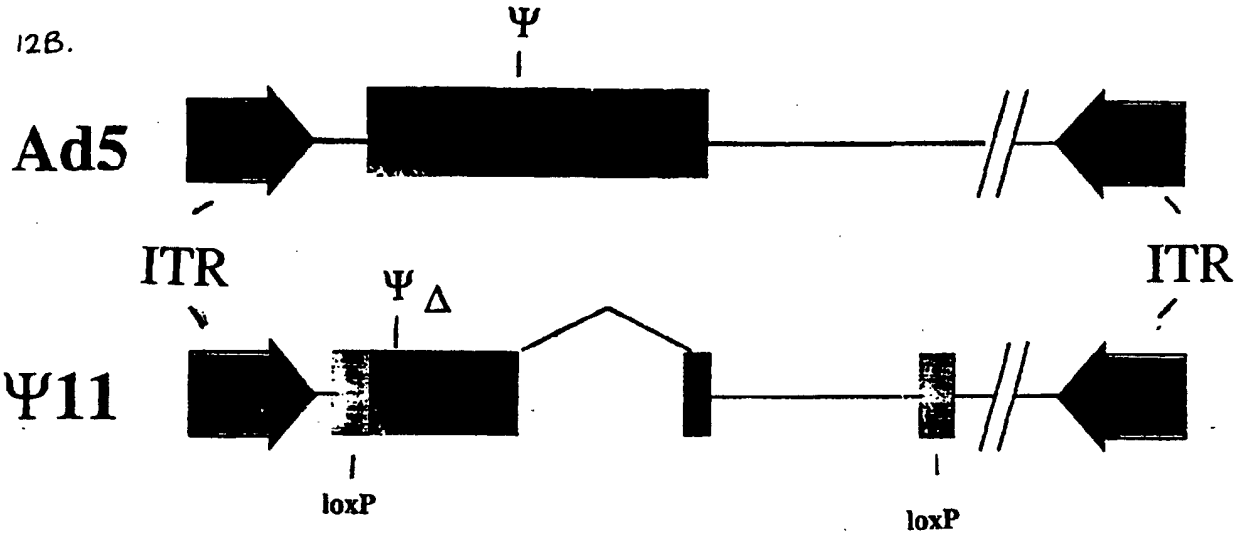
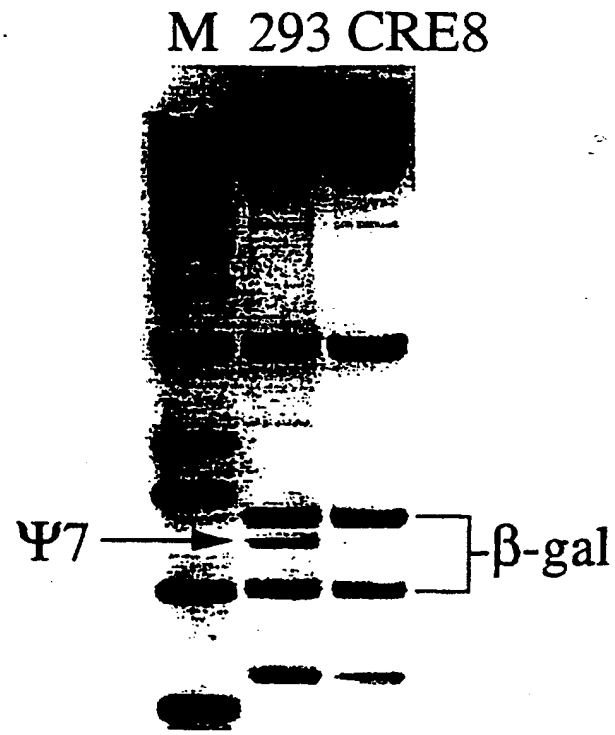


FIG. 12.

Encapsidation Efficiency of Ψ7 in 293 and CRE8 Cells

13A.



13B.

	293	CRE8
β-gal	100	100
Ψ7	31	5.9

FIG. 13.

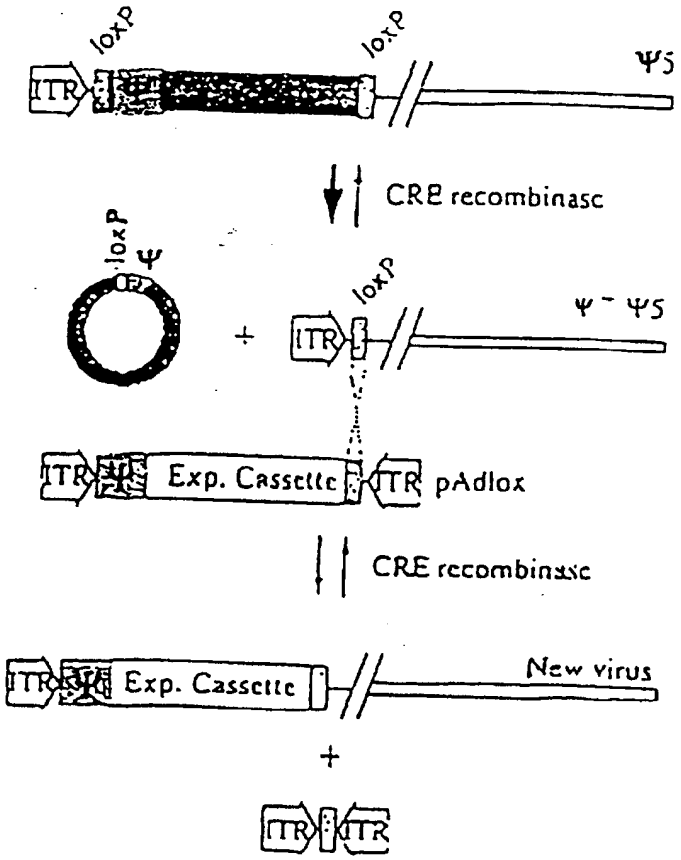

loxP
loxP
Ψ5
ITR
CRE recombinase
loxP
Ψ
ORF
loxP
Ψ - Ψ5
ITR
ITR
Exp. Cassette
ITR
pAdlox
CRE recombinase
ITR
Exp. Cassette
New virus
ITR
ITR

FIG. 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03587

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 63/00; C12N 15/00

US CL :424/93.2; 435/172.3, 320.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.2; 435/172.3, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, EMBASE, MEDLINE, DERWENT BIOTECHNOLOGY, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	FISHER et al. Recombinant Adenovirus Deleted of All Viral Genes for Gene Therapy of Cystic Fibrosis. Virology. 01 March 1996, Vol. 217, pages 11-22, see the entire document.	1, 29 ------ 2-7, 30
P, Y	HAECKER et al. In Vivo Expression of Full-Length Human Dystrophin from Adenoviral Vectors Deleted of All Viral Genes. Human Gene Therapy. 01 October, 1996, Vol. 7, pages 1907-1914, see the entire document.	1-35
P, X	HARDY et al. Construction of Adenovirus Vectors through Cre-lox Recombination. Journal of Virology. March 1997, Vol. 71, No. 3, pages 1842-1849, see the entire document.	1-35

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

13 JUNE 1997

Date of mailing of the international search report

24 JUL 1997

Name and mailing address of the ISA/US
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Authorized officer

BRUCE CAMPELL

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/03587

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, Y	PARKS et al. A helper-dependent adenovirus vector system: Removal of helper virus by Cre-mediated excision of the viral packaging signal. Proceedings of the National Academy of Science, USA. November 1996, Vol. 93, pages 13565-13570, see the entire document.	1-35
P, Y	LIEBER et al. Recombinant Adenoviruses with Large Deletions Genereated by Cre-Mediated Excision Exhibit Different Biological Properties Compared with First-Generation Vectors In Vitro and In Vivo. Journal of Virology. December 1996, Vol. 70, No. 12, pages 8944-8960, see the entire document.	1-35
P, Y	WO 96/40955 A1 (GRAHAM et al.) 19 December 1996, see the entire document.	1-35